A bacterial cell factory for efficient production of ethanol from whey

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Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 August 2017 (31.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/144672 A1

(51) International Patent Classification:
C12P 7/06 (2006.01) *C12N 15/75* (2006.01)

(21) International Application Number:
PCT/EP2017/054347

(22) International Filing Date:
24 February 2017 (24.02.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16157325.8 25 February 2016 (25.02.2016) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

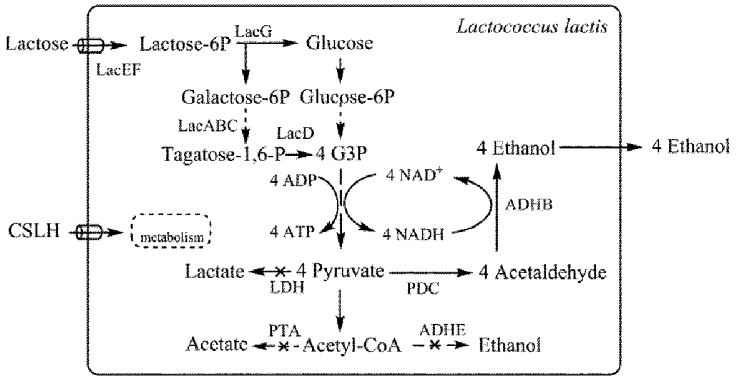
Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: A BACTERIAL CELL FACTORY FOR EFFICIENT PRODUCTION OF ETHANOL FROM WHEY

Figure 1



(57) **Abstract**: The invention relates to a method for homo-ethanol production from lactose using a genetically modified lactic acid bacterium of the invention, where the cells are provided with a substrate comprising dairy waste supplemented with an amino nitrogen source (such as acid hydrolysed corn steep liquor). The invention further relates to genetically modified lactic acid bacterium and its use for homo-ethanol production from lactose in dairy waste. The lactic acid bacterium comprises both genes (*lacABCD, LacEF, lacG*) encoding enzymes catalysing the lactose catabolism pathway; and transgenes (*pdc and adhB*) encoding enzymes catalysing the conversion of pyruvate to ethanol. Additionally a number of genes (*Idh, pta and adhE*) are deleted in order to maximise homo-ethanol production as compared to production of lactate, acetoin and acetate production.

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TITLE:

A BACTERIAL CELL FACTORY FOR EFFICIENT PRODUCTION OF ETHANOL FROM WHEY

Field of the invention

The invention relates to a method for homo-ethanol production from lactose using a genetically modified lactic acid bacterium, where cells of the bacterium are provided with a substrate comprising dairy waste supplemented with an amino nitrogen source, such as hydrolysed corn steep liquor as a source of soluble protein, peptides and free amino acids. Additionally the invention provides the genetically modified lactic acid bacterium that is adapted for homo-ethanol production from lactose in dairy waste, such as whey, deproteinized whey permeate or permeate mother liquor, which is the byproduct after lactose extraction from whey permeate, and for its use for homo-ethanol production. The genetically modified lactic acid bacterium comprises both genes (*lacABCD, lacEF, lacG*) encoding enzymes catalysing a lactose catabolism pathway; and transgenes (*pdc* and *adhB*) encoding enzymes catalysing the conversion of pyruvate to ethanol. The lactic acid bacterium is further genetically modified by inactivation or deletion of those genes in its genome that encode polypeptides having lactate dehydrogenase (EC 1.1.1.27/EC 1.1.1.28); phosphotransacetylase (EC 2.3.1.8), and bifunctional alcohol dehydrogenase (EC 1.1.1.1 and EC 1.2.1.10) activity.

Background of the invention

Currently there is a growing demand for liquid fuels that can be produced in a sustainable manner from renewable raw materials. The potential that lies in using microorganisms for converting various feedstocks, e.g. plant biomass, into useful compounds including fuels, has already been recognized, and intense research is being carried out to establish robust and economically feasible processes for production of biofuels. Microbially produced ethanol presently dominates the biofuel market, and it is mainly produced from either refined sugar or starch derived sugar. Although much focus has been on developing bio-processes, which are reliant on non-food plant biomass as feedstock, there are many challenges, including the high cost of enzymes

needed for degrading the biomass, the recalcitrance of lignocellulose, a lack of microbial catalysts with sufficient robustness to withstand the inhibitors generated in pre-treatment or that have a sufficiently broad spectrum of carbohydrate utilization. As an alternative, one cheap abundant feedstock is cheese whey and its various processed forms, such as whey permeate or whey powder. Whey represents about 85–95% of the milk volume and retains 55% of milk nutrients; and is a liquid byproduct of cheese production, obtained when draining the cheese curd. The worldwide production of cheese whey in 2012 was reported to be 4×10^7 tons and about 50% hereof was used for animal feed or otherwise disposed of as waste. The latter is a serious problem as whey is discarded as liquid waste and has a high BOD (biochemical oxygen demand) and COD (chemical oxygen demand). The composition of whey varies according to the source of the milk and the technology used for its production. Normally it contains approximately 90% water, 4% lactose, 1% protein, 0.7% minerals and small amounts of vitamins. Separation of whey proteins generates whey-permeate and further extraction of lactose leads to permeate mother liquor (residual whey permeate, RWP), which comprises about 60% lactose on a dry weight basis, as a leftover product (Ling, 2008). Fermentation of the main carbon source in whey (lactose) to ethanol has been studied for the last 30 years and most of the research has been focused on yeasts that naturally metabolize lactose. There are, however, problems associated with yeasts, and these include a generally low robustness, slow fermentation-rate, and substrate-inhibition effects, which is why there is a need for better performing microbial candidates.

Lactococcus lactis, which is well-known for its role in cheese production, has great potential as a cell factory, due to properties such as its high glycolytic flux, ability to metabolize a broad range of carbohydrates, well-characterized metabolic network and ease of genetic manipulation. Its long record of safe use is also an important asset, especially for production of food ingredients. Normally, most of the carbon flux in *L. lactis* is directed to lactate (homolactic fermentation). However, it can be successfully engineered to produce ethanol by knocking out alternative product pathways and introducing pyruvate decarboxylase and alcohol dehydrogenase heterologously (Solem et al 2013). A potential drawback of using *L. lactis* as a cell factory is its fastidious nature,

i.e. its many nutritional growth requirements, which could perhaps make it less attractive for some industrial applications, e.g. for production of low-priced chemicals where, for competitive reasons, it is important to keep costs at a minimum. Accordingly, there exists a need to develop cheap fermentation media based on nutrient-rich waste substrates that can circumvent problems associated with using *L. lactis* as a production host organism for ethanol.

Summary of the invention

According to a first embodiment, the invention provides a method for the production of ethanol, comprising the steps of:

a. introducing a genetically modified lactic acid bacterium into an aqueous culture medium;
b. incubating the culture of (a);
c. recovering ethanol produced by said culture in step (b), and optionally
d. isolating the recovered ethanol;

wherein the aqueous culture medium comprises:

I. a lactose rich substrate, such as whey, whey permeate or residual whey permeate, and
II. an amino nitrogen source, such as acid hydrolysed corn steep liquor (CSLH) wherein the concentration of at least one free amino acid, selected from the group consisting on glutamine, histidine, methionine, leucine, isoleucine, and valine in the CSLH is at least 1.5 fold greater than the concentration of the corresponding amino acid in the original corn steep liquor (CAS Number: 66071-94-1) from which the CSLH was derived, and

wherein the genetically engineered lactic acid bacterium comprises transgenes encoding:

i. a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1); and
ii. a polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1); and

wherein the genome of said lactic acid bacterium comprises genes encoding polypeptides having:

iv. lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69)
v. phospho-β-D-galactosidase activity (EC 3.2.1.85)
vi. galactose-6-phosphate isomerase activity (EC 5.3.1.26),
vii. D-tagatose-6-phosphate kinase activity (EC 2.7.1.114), and
viii. tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40);
wherein the genome of said lactic acid bacterium is deleted for genes or lacks genes or lacks functional genes encoding polypeptides having an enzymatic activity of:
ix. lactate dehydrogenase (E.C 1.1.1.27 or E.C.1.1.1.28)
x. phosphotransacetylase (E.C.2.3.1.8) and
xi. bifunctional alcohol dehydrogenase (E.C. 1.1.1.1 and EC 1.2.1.10).

According to a second embodiment, the invention provides for a use of a genetically engineered lactic acid bacterium for the production of ethanol from an aqueous culture medium comprising a lactose rich substrate (such as whey, whey permeate or residual whey permeate), and an amino nitrogen source (such as acid hydrolysed corn steep liquor (CSLH) as defined herein);
wherein the genetically engineered lactic acid bacterium comprises transgenes encoding:

i. a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1); and
ii. a polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1); and
wherein the genome of said lactic acid bacterium comprises genes encoding polypeptides having:
iii. lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69)
iv. phospho-β-D-galactosidase activity (EC 3.2.1.85)
v. galactose-6-phosphate isomerase activity (EC 5.3.1.26),
vi. D-tagatose-6-phosphate kinase activity (EC 2.7.1.114), and
vii. tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40); and
wherein the genome of said lactic acid bacterium is deleted for

genes or lacks genes or lacks functional genes encoding polypeptides having an enzymatic activity of:

viii. lactate dehydrogenase (E.C 1.1.1.27 or E.C.1.1.1.28)
ix. phosphotransacetylase (E.C.2.3.1.8) and
x. bifunctional alcohol dehydrogenase (E.C. 1.1.1.1 and EC 1.2.1.10).

According to a third embodiment, the invention provides the genetically modified lactic acid bacterium for production of ethanol for the production of ethanol from an aqueous culture medium comprising residual a lactose rich substrate (such as whey, whey permeate or whey permeate), and an amino nitrogen source (such as acid hydrolysed corn steep liquor (CSLH)); where the bacterium is as defined above.

Description of the invention

Description of the figures:

Figure 1. Cartoon showing sugar metabolism pathway in *Lactococcus lactis.* The pathway includes *lacEF* genes encoding lactose-specific phosphotransferase system; *lacG* gene encoding phospho-β-galactosidase; *lacAB* genes encoding galactose-6-phosphate isomerase; *LacC gene encoding* D-tagatose-6-phosphate kinase, *lacD* gene encoding tagatose 1,6-diphosphate aldolase, Abbreviations: CSLH, corn steep liquor hydrolysate; AA, amino acids; G3P, glyceraldehyde 3-phosphate; LDH: lactate dehydrogenase, PTA: phosphotransacetylase; ADHE: native alcohol dehydrogenase. PDC: pyruvate decarboxylase and ADHB, alcohol dehydrogenase, are both encoded by codon-optimized synthetic genes based on the *Zymomonas mobilis* sequences. Crosses indicate knock-out of enzyme functions in the genetically modified *L. lactis* strains.

Figure 2. Cartoon showing the genes of the lactose catabolism operon, that is present on the pLP712 plasmid derived from industrial dairy starter strain NCDO712. The plasmid confers on a cell the ability to take up lactose via a lactose-specific phosphotransferase system (PTS), encoded by *lacEF* genes, where after phosphorylated lactose is hydrolyzed to glucose and galactose-6-phosphate (gal-6-P) by the phospho-β-galactosidase (*lacG* gene). The glucose

moiety enters into glycolysis, while gal-6-P is degraded via the tagatose-6-P pathway (*lacABCD* genes).

Figure 3. Graph showing the performance of *L. lactis* strain CS4435L when grown in defined SA medium supplemented with 7.2 g/L lactose (instead of glucose) as the only energy source. The graphs displays: cell density, measured as $OD600_{nm}$ (filled squares); lactose concentration as g/L (filled circles) and ethanol concentration as g/L (filled triangles). The experiments were conducted in duplicate and error bars indicate standard deviations.

Figure 4. Graph showing growth and ethanol production of *L. lactis* strain CS4435L when cultured on residual whey permeate (RWP) alone, or RWP supplemented with the indicated amounts (calculated on a weight per volume basis) of YE, yeast extract; CSL, corn steep liquor; CSLH, corn steep liquor hydrolysate; H1-H3, different hydrolysis conditions (H1: CSL treated with 0.05% H_2SO_4; H2: CSL treated with 0.25% H_2SO_4; H3: CSL treated with 0.5% H_2SO_4). The whey medium comprised three-fold diluted RWP and 50 g/L lactose. After 30 hours in culture, cell growth was determined as final cell density ($OD600_{nm}$); and samples from each culture were collected for measurement of ethanol content. Experiments were conducted at least in duplicate and error bars indicate standard deviations.

Figure 5. Graph showing growth and ethanol production of *L. lactis* strain CS4435L on a medium of only diluted residual whey permeate medium (without the addition of any vitamin or minerals) supplemented with yeast extract (YE) or corn steep liquor hydrolysate (CSLH) as amino-nitrogen sources. (A) The fermentation was carried out in diluted RWP medium containing initially 32 g/L lactose and 0.5% (w/v) yeast extract; (B) The fermentation was carried out in diluted RWP medium containing initially 40 g/L lactose and 2.5% (w/v) CSLH. CSLH was prepared based on H1 conditions (CSL treated with 0.05% H_2SO_4). Samples were collected periodically for determining cell density measured at $OD600_{nm}$ (filled squares), lactose concentration (filled circles) and ethanol concentration (filled triangles) are displayed. Experiments were conducted in duplicate and error bars indicate standard deviations.

Figure 6. Graph showing growth and ethanol production of the *L. lactis* strain CS4435L on diluted residual whey permeate medium containing initially 80 g/L lactose and 2.5% (w/v) CSLH over 55 hours. CSLH was prepared based

on H1 conditions (CSL treated with 0.05% H_2SO_4). Samples were collected periodically for determining cell density measured at $OD600_{nm}$ (filled squares), lactose concentration (filled circles) and ethanol concentration (filled triangles), as displayed. Experiments were conducted in duplicate and error bars indicate standard deviations.

Figure 7. Graph showing growth and ethanol production of the *L. lactis* strain CS4435L during fed-batch culturing. Fed-batch was performed with an initial medium comprising 80 g/L lactose and 2.5% (w/v) CSLH in the residual whey permeate medium, and 500 g/L lactose stock solution was used for feeding. CSLH was prepared based on H1 conditions (CSL treated with 0.05% H_2SO_4). Samples were collected periodically for determining cell density (filled squares), lactose concentration (filled circles) and ethanol concentration (filled triangles) as displayed. Experiments were conducted in duplicate and error bars indicate standard deviations.

Figure 8. Graph showing ethanol production by the *L. lactis* strain CS4435L when cultured in 3 different growth media, each comprising diluted residual whey permeate medium and 80 g/L lactose and supplemented with one of: (1) 2.5% (w/v) CSLH prepared under H1 conditions (CSL treated with 0.05% H_2SO_4); (2) 2.5% (w/v) CSL and 70 IU/L proteinase; and (3) 2.5% (w/v) CSL and 700 IU/L proteinase (*Aspergillus melleus*). Samples were collected periodically for determining ethanol concentration (filled triangles), over a period of 40 -50 hours as displayed.

Abbreviations and terms:

Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. This sequence identity obtained using the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Sequence alignment is performed

with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: Proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

Corn steep liquor: has CAS Number: 66071-94-1 and is a by-product of corn wet-milling industry. It contains proteins, amino acids, vitamins and minerals and contains approx. 50% (w/w) solids.

Deleted gene: the deletion of a gene from the genome of a bacterial cell leads to a loss of function of the gene and hence where the gene encodes a polypeptide the deletion results in a loss of expression of the encoded polypeptide. Where the encoded polypeptide is an enzyme, the gene deletion leads to a loss of detectable enzymatic activity of the respect polypeptide in the bacterial cell.

Functional gene: gene that is capable of expressing an active enzyme encoded by the gene. Loss of a gene or loss of the function of a gene results in an inability to express the active enzyme encoded by the gene. A loss of function may be the result of a failure to transcribe the gene; or may be a failure to translate the transcribed gene into an active enzyme (e.g. due to mutations). When an enzyme loses more than 60% activity, preferably more than 90% activity; it is deemed to be inactive, in the sense that it no longer has a significant influence on product flux in the sugar metabolism pathway.

gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences

from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

Native gene: endogenous gene in a bacterial cell genome (where the genome includes plasmids), where the gene is homologous to the host bacterium.

Transgenes encoding polypeptides having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1) and alcohol dehydrogenase (ADHB) activity (EC 1.1.1.1) confer on a cell the ability to convert pyruvate to ethanol via acetaldehyde.

Whey and whey permeate and residual whey permeate: whey is a byproduct of cheese manufacture; and comprises whey proteins having a high nutritional value and lactose. Removal of whey proteins, typically by means of ultrafiltration or diafiltration produces a whey protein concentrate and whey permeate that is lactose-rich. The lactose content of the whey permeate is dependent on the treatment conditions and typically it can reach as high as hundreds of grams per liter by reverse osmosis, such as 200 g/L. Removal of fat from whey, or from lactose-rich permeate, typically by centrifugation, yields a fat-free composition (whey or permeate). Residual whey permeate (also called permeate mother liquor) is obtained after the extraction of lactose from whey permeate (typically by lactose crystallisation); and has a lower lactose content of about 150 g/L.

Detailed description of the invention

I: A genetically modified lactic acid bacterium for the production of ethanol from lactose

Lactococcus lactis is a homo-lactic fermentative lactic acid bacterium, directing about 90% of metabolic flux to lactate when grown on fast fermentable sugars, such as glucose (Figure 1). The wild-type *L. lactis* strain MG1363 exemplifies the homolactic fermentation of *L. lactis* when grown on glucose, as shown in Table 3 of Example 1. *L. lactis* is naturally capable of producing ethanol, but only in small amounts. Thus a number of genetic modifications are required to redirect *L. lactis* metabolism towards homo-ethanol production, as described below.

In order to limit metabolic flux towards lactate in the lactic acid bacterium of the invention, the activity of the enzymes of the lactate pathway are reduced by inactivating or deleting one or more genes, for example *ldh, ldhX* and *ldhB,* encoding enzymes of this pathway. In order to additionally prevent metabolic flux to acetate, the activity of the acetate pathway is reduced by inactivating or deleting the gene encoding phosphotransacetylase (*pta*), which converts acetylphosphate to acetate. Additionally, the native alcohol dehydrogenase gene (*adhE*) encoding a bifunctional alcohol dehydrogenase, ADHE (EC 1.1.1.1 and EC 1.2.1.10) may be inactivated or deleted in order to maximize the production of ethanol with a high yield. So long as the native alcohol dehydrogenase (ADHE) is active, another byproduct (acetoin) is formed in order to balance the cofactors (2 NADH is formed per glucose by glycolysis, while 4 NADH is required for the complete reduction to ethanol by ADHE, which means only half of the carbon flux can be diverted to ethanol). Therefore, the removal of ADHE activity is beneficial for high yield ethanol production.

The lactic acid bacterium of the invention, further comprises codon-optimized transgenes (*pdc* and *adhB*), sourced from *Zymomonas moblis*, encoding pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) enzymes, respectively. Expression of these encoded PDC and ADHB enzymes in cells of the lactic acid bacterium of the invention, where the lactate and acetate pathways are inactivated or deleted, confers the ability for homo-ethanol production and for growth under anaerobic growth conditions (see Strain CS4435 in Table 3 of Example 1). The above described genetic modifications in the lactic acid bacterium of the invention provide the cells with a metabolic pathway for the use of (extracellular) glucose as substrate for homo-ethanol production.

The lactic acid bacterium of the invention, however, further comprises genes encoding enzymes that facilitate the uptake of extracellular lactose and its entry into the glycolytic pathway; such that lactate can be used as substrate for homo-ethanol production. Preferably, the cells of the lactic acid bacterium comprise the following genes: a gene encoding a lactose specific phosphotransferase system (PTS) e.g., a *lacEF* gene, whereby phosphorylated

lactose is assimilated by the cells; a gene encoding a phospho-β-galactosidase (e.g. a *lacG* gene) that hydrolyzes lactose phosphate to glucose and galactose-6-phosphate (gal-6-P), where the glucose moiety enters into glycolysis; genes encoding the tagatose-6-P pathway, e.g., the *lacAB, lacC, lacD* genes encoding galactose-6-phosphate isomerase, D-tagatose-6-phosphate kinase, and tagatose 1,6-diphosphate aldolase, respectively, whereby gal-6-P is degraded and enters the glycolytic pathway as glyceraldehyde-3-phosphate.

Cells of the lactic acid bacterium of the invention are shown to be efficient producers of ethanol from lactose via homo-ethanol fermentation with a growth rate that is close to that on a glucose-containing medium (Example 1).

The characteristics of the individual genes that may be deleted or introduced in order to produce a lactic acid bacterium of the invention are detailed below.

I.i Deletion of the lactate pathway: The lactic acid bacterium of the invention is characterised by knockouts of one or more endogenous native gene encoding a polypeptide having lactate dehydrogenase activity causing a block in the lactate synthesis pathway in the bacterium. Deletion of at least one gene (e.g. *ldh*) encoding a lactate dehydrogenase enzyme (E.C 1.1.1.27 or EC 1.1.1.28) provides a lactic acid bacterium of the invention that is depleted in lactate production. For example, where the lactic acid bacterium of the invention belongs to a given genus, the deleted endogenous gene is one encoding a polypeptide having lactate dehydrogenase activity in that genus. Preferably the polypeptide having lactate dehydrogenase activity (EC 1.1.1.27 or EC 1.1.1.28) has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% amino acid sequence identity to one of the following sequences: SEQ ID NO: 2 in a *Lactococcus* species (e.g. *Lactococcus lactis*); SEQ ID NO: 4, 6, or 8 in a *Lactobacillus* species (e.g. *Lactobacillus acidophilus*); SEQ ID NO: 10 in a *Lactobacillus* species (e.g. *Lactobacillus delbrueckii*); SEQ ID NO. 12, 14 or 16 in a *Lactobacillus* species (e.g. *Lactobacillus casei*), SEQ ID NO. 18 or 20 in a *Lactobacillus* species (e.g. *Lactobacillus plantarum*); SEQ ID NO: 22 in a *Pediococcus* species *(e.g. Pediococcus pentosaceus*), SEQ ID NO: 24 or 26 in a *Leuconostoc* species

(e.g. Leuconostoc mesenteroides), SEQ ID NO: 28 in a *Streptococcus* species *(e.g. Streptococcus thermophilus),* SEQ ID NO: 30 or 32 in a *Oenococcus* species *(e.g. Oenococcus oeni),* and SEQ ID NO: 34 or 36 in a *Bacillus* species *(e.g. Bacillus coagulans*).

In one embodiment, an additional endogenous gene, encoding a polypeptide having lactate dehydrogenase enzymatic activity (E.C 1.1.1.27 or EC1.1.1.28), is deleted from the lactic acid bacterium of the invention. For example, where the lactic acid bacterium of the invention belongs to the genus *Lactococcus,* the deleted gene (*ldhX*) encodes a polypeptide having at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% amino acid sequence identity to SEQ ID NO: 38.

In one embodiment, an additional endogenous gene, encoding a polypeptide having lactate dehydrogenase enzymatic activity (EC 1.1.1.27 or EC 1.1.1.28), is deleted from the lactic acid bacterium of the invention. For example, where the lactic acid bacterium of the invention belongs to the genus *Lactococcus,* the deleted gene (*ldhB*) encodes a polypeptide having at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% amino acid sequence identity to SEQ ID NO: 40. Further, where the lactic acid bacterium of the invention belongs to the genus *Lactococcus,* the three genes (*ldh, ldhB* and *ldhX*) encoding a polypeptide having at least 70% amino acid sequence identity to SEQ ID NO: 2, 38 and 40 respectively may be deleted.

I.ii Deletion of the acetate pathway: In one embodiment, the lactic acid bacterium of the invention is characterised by knockout of the endogenous native gene encoding a phosphotransacetylase (EC 2.3.1.8), causing a block in the acetate synthesis pathway in the bacterium. Deletion of a gene (e.g. *pta*) encoding a phosphotransacetylase enzyme provides a lactic acid bacterium of the invention that is blocked in acetate production. For example, where the lactic acid bacterium of the invention belongs to a given genus, the deleted endogenous gene is one encoding a polypeptide having phosphotransacetylase activity (EC 2.3.1.8) in that genus. Preferably the polypeptide having phosphotransacetylase activity has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% amino acid sequence

identity to one of the following sequences: SEQ ID NO: 42 in a *Lactococcus* species (e.g. *Lactococcus lactis*); SEQ ID NO: 44, 46, 48, and 50 in a *Lactobacillus* species (e.g. *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus plantarum*), SEQ ID NO: 52 in a *Pediococcus* species (e.g. *Pediococcus pentosaceus*), SEQ ID NO: 54 in a *Leuconostoc* species (e.g. *Leuconostoc mesenteroides*), SEQ ID NO: 56 in a *Streptococcus* species (e.g. *Streptococcus thermophilus*), SEQ ID NO: 58 *Oenococcus* species (e.g. *Oenococcus oeni*), and SEQ ID NO: 60 in a *Bacillus* species (e.g. *Bacillus coagulans*).

I.iii Deletion of the ethanol pathway: In one embodiment, the lactic acid bacterium of the invention is characterised by knockout of the endogenous native gene encoding bifunctional alcohol dehydrogenase activity (EC 1.1.1.1 and EC 1.2.1.10) causing a block in the ethanol synthesis pathway in the bacterium. Deletion of the gene encoding an alcohol dehydrogenase enzyme provides a lactic acid bacterium of the invention that is blocked in ethanol production.

For example, where the lactic acid bacterium of the invention belongs to a given genus, the deleted endogenous gene (e.g. *adhE*) is one encoding a polypeptide having bifunctional alcohol dehydrogenase activity (EC 1.1.1.1 and EC 1.2.1.10) in that genus. Preferably the polypeptide having alcohol dehydrogenase activity has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% amino acid sequence identity to one of the following sequences: SEQ ID NO: 62 in a *Lactococcus* species (e.g. *Lactococcus lactis*); SEQ ID NO: 64 in a *Lactobacillus* species (e.g. *Lactobacillus acidophilus*); SEQ ID NO: 66 or 68 in a *Lactobacillus* species (e.g. *Lactobacillus casei*); SEQ ID NO: 70 in a Lactobacillus species (e.g., *Lactobacillus plantarum*), SEQ ID NO: 72 in a *Leuconostoc* species (e.g. *Leuconostoc mesenteroides*), SEQ ID NO: 74 in a *Streptococcus* species (e.g. *Streptococcus thermophilus*), SEQ ID NO: 76 in a *Oenococcus* species (e.g. *Oenococcus oeni*), and SEQ ID NO: 78 in a *Bacillus* species (e.g. *Bacillus coagulans*).

I.iv Transgene encoding an enzyme having pyruvate decarboxylase activity: The bacterium of the invention comprises a transgene encoding a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1) that converts pyruvate to acetaldehyde. The amino acid sequence of the polypeptide has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the pyruvate decarboxylase (SEQ ID NO: 80) encoded by a codon optimized derivative of the *Zymomonas mobilis pdc* gene.

I.v Transgene encoding an enzyme having alcohol dehydrogenase activity: The bacterium of the invention comprises a transgene encoding a polypeptide having alcohol dehydrogenase (ADH) activity (EC 1.1.1.1) that converts acetaldehyde to ethanol, but is not able to use acetyl-CoA as substrate (the acetaldehyde being formed by pyruvate decarboxylase activity). The expression of these heterologous enzymes, PDC and ADHB, enables the complete cofactor balance thereby facilitating maximum ethanol production. The amino acid sequence of the polypeptide has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the pyruvate decarboxylase (SEQ ID NO: 82) encoded by a codon optimized derivative of the *Zymomonas mobilis adhB* gene.

I.vi Genes encoding enzymes of the lactose catabolism pathway:

The lactic acid bacterium of the invention comprises the following native genes or transgenes required for lactose assimilation and catabolism:
1) a first and second gene encoding a first and a second polypeptide component together conferring lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69), whereby phosphorylated lactose is assimilated by the cells. The amino acid sequence of the first polypeptide component has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the phosphotransferase system EIICB component (SEQ ID NO: 84) encoded by the *Lactococcus lactis lacE* gene; and the amino acid sequence of the second polypeptide component has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence

of the phosphotransferase system EIIA component (SEQ ID NO: 86) encoded by the *Lactococcus lactis lacF* gene; and

2) a gene encoding a polypeptide having phospho-β-D-galactosidase activity (EC 3.2.1.85) that hydrolyzes lactose-6-phosphate to glucose and galactose-6-phosphate (gal-6-P), whereby the glucose moiety can then enter the glycolytic pathway. The amino acid sequence of the polypeptide has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the phospho-β-D-galactosidase (SEQ ID NO: 88) encoded by the *Lactococcus lactis lacG* gene.

Additionally, the following genes encoding enzymes in the tagatose-6-P pathway, whereby gal-6-P is degraded and enters the glycolytic pathway as glyceraldehyde-3-phosphate, are required:

3) a first and second gene encoding a first and a second polypeptide subunit together conferring galactose-6-phosphate isomerase activity (EC 5.3.1.26). The amino acid sequence of the first polypeptide subunit has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the first subunit of the galactose-6-phosphate isomerase (SEQ ID NO: 90) encoded by the *Lactococcus lactis lacA* gene; and the amino acid sequence of the second polypeptide subunit has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the second subunit of the galactose-6-phosphate isomerase (SEQ ID NO: 92) encoded by the *Lactococcus lactis lacB* gene; and

4) a gene encoding a polypeptide having D-tagatose-6-phosphate kinase activity (EC 2.7.1.114). The amino acid sequence of the polypeptide has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the D-tagatose-6-phosphate kinase (SEQ ID NO: 94) encoded by the *Lactococcus lactis lacC* gene; and

5) a gene encoding a polypeptide having tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40). The amino acid sequence of the polypeptide has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100%

sequence identity to the amino acid sequence of the tagatose 1,6-diphosphate aldolase (SEQ ID NO: 96) encoded by the *Lactococcus lactis lacD* gene; and

6) optionally a gene encoding a polypeptide having lactose transport regulator activity. The amino acid sequence of the polypeptide has at least 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% sequence identity to the amino acid sequence of the lactose transport regulator (SEQ ID NO: 98) encoded by the *Lactococcus lactis lacR* gene.

I.vii Fermentation properties of the lactic acid bacterium genetically modified for ethanol production

Lactococcus lactis is characterised by homo-lactic fermentation when grown on glucose (Figure 1), as illustrated for the wild-type *L. lactis* strain MG1363 in Table 3. *L. lactis* strain CS4435 derived from MG1363 by deletion of the lactate dehydrogenase genes (*ldh ldhB, ldhX*); the phosphotransacetylase gene (*pta*); and the alcohol dehydrogenase gene (*adhE*); and expressing the pyruvate decarboxylase gene (*pdc*) and ethanol dehydrogenase gene (*adhB*) derived from *Zymomonas mobilis*; is characterised by homo-ethanol fermentation when grown on glucose (Table 3).

The strain CS4435L, derived from strain CS4435, comprises and expresses the entire lactose catabolism pathway, encoded by genes on the *Lactococcal* plasmid, pLP712 (55.395 kbp) (figure 2). Strain CS4435L is capable of growth on a defined medium with lactose as the sole energy source, at a growth rate of 0.6 h^{-1}, close to that on glucose as sole energy source. The sole detected fermentation product of this strain was ethanol, with an ethanol titer of 3.2 g/L, and a yield of 0.45 g ethanol/g lactose, corresponding to 83% of the theoretical maximum. Higher ethanol titers, of up to 12.0 g/L, were achieved by increasing the initial lactose concentration in the growth medium (Table 2).

II A genetically modified lactic acid bacterium comprising a pathway for homo-ethanol production from lactose in diary waste

The lactic acid bacterium according to the invention, that comprises genes encoding a pathway for homo-ethanol production, as described in section I, is

a member of a genus of lactic acid bacteria selected from the group consisting of *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc, Streptococcus, Oenococcus,* and *Bacillus,* preferably *Lactococcus*. The lactic acid bacterium of the invention may for example be a species of lactic acid bacteria selected from the group consisting of *Lactococcus lactis, Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus plantarum, Pediococcus pentosaceus, Leuconostoc mesenteroides, Streptococcus thermophilus, Oenococcus oeni* and *Bacillus coagulans.*

III A sustainable and economical bioprocess for ethanol production using the genetically modified lactic acid bacterium of the invention

Residual whey permeate (RWP) is the permeate mother liquor after extracting lactose from whey permeate, and is a waste product of the dairy industry. However, its use as a feedstock for ethanol production has the potential to create a sustainable and economical bioprocess for ethanol production. RWP comprises lactose, as a source of energy, as well as the amino acids essential for *L. lactis* growth, although in relatively low concentrations (Table 5).

A growth medium, based on RWP alone, was found insufficient to support either fermentative growth or ethanol production by the genetically modified lactic acid bacterium of the invention (Example 2). A supplement to the RWP medium to provide a source of complex amino nitrogen was found essential, since fermentative growth and ethanol production were obtained when a supplement of yeast extract (0.5% w/v) was provided, while ammonium salts were insufficient (Example 2).

The use of YE as a supplement to RWP, while effective, does not provide a cost-effective growth medium for producing ethanol using lactic acid bacteria, for a number of reasons, as explained herein. Firstly, due to its high price (currently 7000~10000 $/ton), it would increase the total ethanol production costs using the genetically modified lactic acid bacterium of the invention on RWP supplemented media by an estimated 30% , and for this reason alone there exists a need to find cheaper alternative sources of complex amino nitrogen.

Corn steep liquor having CAS Number: 66071-94-1 (CSL), which is a byproduct of the corn milling industry, is a cheaper amino nitrogen source that currently costs around 500 $/ton. CSL has a pH of about 4.0 and consists predominantly of naturally occurring nutritive materials such as water-soluble proteins, amino acids (e.g., alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, valine), vitamins (e.g., B-complex), carbohydrates, organic acids (e.g., lactic acid), minerals (e.g., Mg, P, K, Ca, S), enzymes and other nutrients. However, even though CSL is a source of complex amino nitrogen, when the genetically modified lactic acid bacterium of the invention was supplied with the RWP medium supplemented with CSL in an amount corresponding to 0.1% to 2.5% (w/v)), the final biomass concentration obtained was still very low and almost no ethanol was produced.

Surprisingly, the hydrolysis of CSL, for example acid hydrolysis, yielded a hydrolysed CSL composition (CSLH) that when used as a supplement to RWP provided an effective low cost medium for ethanol production by fermentation using the genetically modified lactic acid bacterium of the invention (Table 4, in Example 2). The need to use hydrolysed CSL as a supplement was unexpected, since many native strains of lactic acid bacteria, including the strain CS4435L used in the examples, comprise a cell-envelope bound protease enabling their growth on milk, which is also low in free amino acids and peptides. Only limited acid hydrolysis was required to increase the content of free peptides and amino acids in the CSL, sufficient to produce a suitable CSLH supplement. The growth and high yield of ethanol produced by the genetically modified lactic acid bacterium of the invention on RWP supplemented with the CSLH (obtained by limited acid hydrolysis) is consistent with the fact that cells of these lactic acid bacteria comprise both intracellular peptidases as well as various uptake systems for peptides as well as free amino acids that facilitate the assimilation of amino nitrogen in this form.

Alternative methods for improving growth and ethanol production were also tested using RWP supplemented with treated-forms of CSL (see example 4, figure 8). Surprisingly, growth media comprising acid-hydrolyzed CSLH supported significantly higher levels of ethanol production than proteinase-

treated CSL. Thus, *L. lactis* strain CS4435L cultures grown in media supplemented with CSLH produced 24 g/L ethanol in 39 h; while cultures grown in media supplemented with 70 IU/L or 700 IU/L proteinase-treated CSL, only produced 8.9 g/L and 10.7 g/L ethanol in 48 h, respectively.

Down-stream processing of ethanol produced by fermentation, typically involves a distillation step. In order to minimize distillation costs it is important that the ethanol content of the fermentation broth is at least 4% (w/w) ethanol. Employing a fed-batch fermentation process, a fermentation broth comprising over 4.1% (w/w) ethanol was obtained when the genetically modified lactic acid bacterium of the invention was cultured on RWP supplemented with CSLH, and subsequent feeding with lactose (Example 3, and Table 4).

Accordingly, the invention provides a method for ethanol production employing a genetically engineered lactic acid bacterium comprising the steps of:

a. introducing a genetically modified lactic acid bacterium into an aqueous culture medium;
b. incubating the culture of (a);
c. recovering ethanol produced by said culture in step (b), and optionally
d. isolating the recovered ethanol;
wherein the aqueous culture medium comprises a lactose rich substrate (such as whey permeate or residual whey permeate), and a amino nitrogen source (such as acid hydrolysed corn steep liquor, as defined herein), and
wherein the genetically engineered lactic acid bacterium comprises transgenes encoding:
- a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1; and
- a polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1);
wherein the genome of said lactic acid bacterium is deleted for genes or lacks genes or lacks functional genes encoding polypeptides having an enzymatic activity of:
- lactate dehydrogenase (E.C 1.1.1.27 or E.C.1.1.1.28)
- phosphotransacetylase (E.C.2.3.1.8) and

- bifunctional alcohol dehydrogenase E (E.C. 1.1.1.1 and EC 1.2.1.10); and

wherein the the genome of said lactic acid bacterium comprises genes encoding polypeptides having:

- lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69)
- phospho-β-D-galactosidase activity (EC 3.2.1.85)
- galactose-6-phosphate isomerase activity (EC 5.3.1.26),
- D-tagatose-6-phosphate kinase activity (EC 2.7.1.114), and
- tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40);
- and optionally a lactose transport regulator protein.

According to one embodiment of the method for ethanol production according to the invention, the aqueous culture medium comprises whey permeate or residual whey permeate, combined with acid hydrolysed corn steep liquor: wherein the final lactose content of the culture medium is at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 g/L lactose; between any one of 2 - 200 g/L, 5 - 180 g/L, 5 - 150 g/L, 5 - 100 g/L, 10 - 100 g/L, 5 - 90 g/L, 10 - 90 g/L, 5 - 80 g/L, 10 - 80 g/L lactose; preferably equal to or less than any one of 150 g/L, 140 g/L, 130 g/L, 120 g/L, 110 g/L, 100 g/L, 90 g/L, and 80 g/L lactose. The desired lactose content of the aqueous culture medium is obtainable by diluting the whey permeate of residual whey permeate in the aqueous culture medium. For example, the aqueous culture medium may comprise any one of 1-80%, 5-80%, 10 – 80%, 20 – 80%, 20 – 60%, and 30-50% residual whey permeate.

The hydrolysed corn steep liquor (CSLH) component of the aqueous culture medium, is derived from corn steep liquor (CAS Number: 66071-94-1) by acid hydrolysis, for example hydrolysis with H_2SO_4, or HCl. The hydrolysed CSL is characterized by enhanced levels of soluble proteins, peptides and free amino acids (the concentrations of nearly all the 20 free amino acids is doubled compared with untreated corn steep liquor, especially arginine, glutamine, histidine, methionine, isoleucine, leucine, valine, and tyrosine. The peptides present in CSLH include oligopeptides of 2, 3, 4, 5 amino acid residues or even longer peptides; in particular the peptides Leu-Gly, Gly-Gly, Gly-Gly-Leu, Thr-Pro-Val-Gly-Lys.

A hydrolysed CSLH preparation, suitable for use as a supplement to a RWP medium, is one wherein the concentration of at least one free amino acid, selected from the group consisting on glutamine, histidine, methionine, leucine, isoleucine, and valine is at least 1.5 fold greater than the concentration of the corresponding amino acid in the original CSL from which the CSLH was derived by hydrolysis. Preferably, the concentration of the at least one free amino acid is at least 1.6, 1.7, 1.8, 1.9 or 2 fold greater than the concentration of the corresponding amino acid in the original CSL from which the CSLH was derived by hydrolysis. When the at least one free amino acid is histidine, the concentration of the histidine in a CSLH preparation having a 25% w/v solids content is at least 8mM, preferably at least 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20mM; while the mM concentration of the histidine in a 2.5% (w/v solids content) CSLH preparation is correspondingly 10 folded lower. Alternatively, when the at least one free amino acid is histidine, the concentration of the histidine in a CSLH preparation having a 25% w/v solids content is at least 2mM, preferably at least 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 mM higher than the concentration of histidine in the original CSL from which the CSLH was derived by hydrolysis; while the mM concentration of the histidine in a 2.5% (w/v solids content) CSLH preparation is correspondingly 10 folded lower.

The RWP medium growth is supplemented with CSLH in an amount sufficient to support ethanol production using the genetically modified lactic acid bacterium of the invention. It has been observed that addition of acid hydrolysed treated CSL (H1) to give a final concentration of at least 0.5 w/v solids content was sufficient to enhance ethanol production and growth. Preferably, when the initial lactose concentration of the growth medium is between 5 - 80 g/L lactose, then the acid hydrolysed CSL (H1) is added to the medium in an amount to give a final w/v CSL solids concentration of at least 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.5, 2.75, 3.0%. Preferably, when the initial lactose concentration of the growth medium is between 60 - 200 g/L lactose, then acid hydrolysed CSL (H1) is added in an amount to give a final CSL w/v solids concentration of at least 2.00, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75 and 5.0%. The desired final concentration of CSLH in the aqueous culture medium is obtainable by diluting

the CSLH into the aqueous culture medium. CSL is obtainable in a CSL w/v solids concentration of 40 to 60%; typically 50%. Accordingly, a dilution of 10 fold into the aqueous culture medium will give a CSL w/v solids concentration of 4 to 6%; typically 5%. Since the solids content of CSL remains unaltered by the acid hydrolysis, the final dilution of CSLH required to obtain the desired final concentration are the same as those for the original CSL from which the CSLH was derived.

According to one embodiment of the method for ethanol production according to the invention, the aqueous culture medium may be further characterized as a composition consisting of the components: whey permeate of residual whey permeate (as defined herein); hydrolysed corn steep liquor (as defined herein); water and optionally supplemented with yeast extract and/or an aqueous solution of lactose.

The ethanol produced by fermentation using the genetically modified lactic acid bacterium of the invention can be recovered from the fermentation medium by steps including distillation.

IV A method of detecting ethanol production

Methods for detecting and quantifying ethanol produced by a micro-organism of the invention include high performance liquid chromatography (HPLC) combined with Refractive Index detection to identify and quantify ethanol (as described by Solem et al., 2013) relative to a standard, as described and illustrated in the examples.

V Methods for producing a lactic acid bacterium of the invention

Integration and self-replicating vectors suitable for cloning and introducing one or more gene encoding one or more a polypeptide having an enzymatic activity required for homo-ethanol production in a lactic acid bacterium of the invention are commercially available and known to those skilled in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Cells of a lactic acid bacterium are genetically engineered by the introduction into the cells of

heterologous DNA (RNA). Heterologous expression of genes encoding one or more polypeptide having an enzymatic activity required for homo-ethanol production in a lactic acid bacterium of the invention is demonstrated in Example 1.

A nucleic acid molecule, that encodes one or more polypeptide having an enzymatic activity required for homo-ethanol production according to the invention, can be introduced into a cell or cells and optionally integrated into the host cell genome using methods and techniques that are standard in the art. For example, nucleic acid molecules can be introduced by standard protocols such as transformation including chemical transformation and electroporation, transduction, particle bombardment, etc. Expressing the nucleic acid molecule encoding the enzymes of the claimed invention may also be accomplished by integrating the nucleic acid molecule into the genome.

Deletion of endogenous genes in a host lactic acid bacterium to obtain a genetically modified lactic acid bacterium according to the invention can be achieved by a variety of methods; for example by transformation of the host cell with linear DNA fragments containing a locus for resistance to an antibiotic, or any other gene allowing for rapid phenotypic selection, flanked by sequences homologous to closely spaced regions on the cell chromosome on either side of the gene to be deleted, in combination with the immediate subsequent deletion or inactivation of the recA gene. By selecting for a double-crossover event between the homologous sequences, shown by the antibiotic resistance or other detectable phenotype, a chromosome disruption can be selected for which has effectively deleted an entire gene. Inactivation or deletion of the recA gene prevents recombination or incorporation of extrachromosomal elements from occurring, thereby resulting in a bacterial strain which is useful for screening for functional activity or production of genetically engineered proteins in the absence of specific contaminants. The deletion of the genes *ldh, ldhB, ldhX, pta, and adhE* from *L. lactis* given in Example 1, illustrates one method for deleting these genes.

The deletion of endogenous genes in a host lactic acid bacterium to obtain a genetically modified lactic acid bacterium according to the invention can also

be achieved by the more traditional approach involving mutagenesis and screening/selection. For instance, LDH (lactate dehydrogenase) mutants can be screened out using solid medium containing 2,3,5-triphenyl tetrazolium following mutagenesis using for instance N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or UV radiation. Alternatively, after mutagenesis, low-acid producing strains could be selected using a combination of bromide and bromate as described by Han et al., 2013. ALDB (α-acetolactate decarboxylase) mutants can be obtained easily after mutagenesis, for instance using NTG, or grown in the medium containing an unbalanced concentration of leucine versus valine and isoleucine in the medium (Goupil et al., 1996). ADHE (ethanol dehydrogenase) mutants can be screened in the presence of various concentrations of acetaldehyde.

Examples

Example 1 Genetic modification of a *Lactococcus lactis* strain for production of ethanol from lactose

The genetic modifications required to produce a *Lactococcus lactis* strain that is capable of homo-ethanol production from lactose in dairy waste and to efficiently direct the flux towards this end product are described below.

1.1 Host strains and plasmids:

The plasmid-free strain *Lactococcus lactis subsp. cremoris* MG1363 or its derivatives were used for the studies described herein [18]. The *Escherichia coli* strain ABLE-C (*E. coli* C lac (LacZ-)[Kan^r McrA$^-$ McrCB$^-$ McrF$^-$ Mrr$^-$ HsdR (r_k^- m_k^-)][F´proAB lacI^{q}ZΔM15 Tn10(Tetr)]) (Stratagene) was used only for facilitating DNA cloning steps. The lactose-metabolism plasmid pLP712 (55,395 bp) was extracted from the dairy isolate NCDO712 based on the method of Andersen (1983).

1.2 Growth conditions:

E. coli strains were grown aerobically at 30°C in Luria-Bertani broth (Sambrook et al. 2001). For growth experiments *L. lactis* was grown in 100 ml flasks without shaking in defined SA medium (Jensen et al., 1993), where glucose was replaced by lactose, or residual whey permeate medium (RWP). RWP, which was provided from Arla Foods Ingredients Group P/S

(http://www.arlafoodsingredients.com/), is the mother liquor from lactose production and its composition can be seen in Table 3. When required, yeast extract (Sigma-Aldrich, USA) was used as an amino nitrogen source. Antibiotics were added in the following concentrations: erythromycin: 200 µg/ml for *E. coli* and 5 µg/ml for *L. lactis*, tetracycline: 8 µg/ml for *E. coli* and 5 µg/ml for *L. lactis*, chloramphenicol: 20 µg/ml for E. coli and 5 µg/ml for *L. lactis*.

For nitrogen source optimization tests, 50 g/L lactose (diluted RWP) was mixed with different concentrations of nitrogen sources (NH_4Cl, yeast extract, CSL or CSLH) in 25 ml tube with a volume of 10 ml.

For ethanol production tests, RWP was diluted and used as the main substrate for fermentation without the addition of any vitamins or salts, except 2.5% (w/v) CSLH. *L. lactis* strain CS4435L was grown in a 125 ml flask with 100 ml of medium with slow magnetic stirring and no aeration. The cultivation was carried out at 30°C.

1.3 DNA techniques:

All manipulations were performed according to Sambrook et al (1989). PCR primers used can be seen in Table 2. PfuX7 polymerase was used for PCR applications (Nørholm, 2010). Chromosomal DNA from *L. lactis* was isolated using the method described for *E. coli* with the modification that cells were treated with 20 µg of lysozyme per ml for 2 hours. Cells of *E. coli* were transformed using electroporation. *L. lactis* was made electro competent as described previously by Holo and Nes (1989), with the following modifications: the cells were grown with 1% glycine, and at an optical density of 0.5 (600 nm) ampicillin was added to a final concentration of 20 µg ml^{-1} and incubation was continued at 30°C for 30 minutes.

The plasmid vector pCS1966 (Solem et al., 2008) was used for deleting genes in *L. lactis*. Plasmids employed for deleting chromosomal genes were prepared by PCR amplifying approximately 800 base pairs (bp) regions upstream and downstream of the *L. lactis* chromosomal region to be deleted using the PCR primers and chromosomal DNA isolated from *L. lactis*. The primers used for amplifying the upstream and downstream regions are indicated in Table 2 as "geneX ups." and geneX dwn". The amplified fragments and the plasmid,

pCS1966, were then digested with the respective restriction enzymes indicated in the primer table, prior to inserting the fragment into the plasmid. The resulting plasmids were transformed into the parent strain individually and gene deletion was performed as described by Solem et al., (2008). Specifically, the plasmids were transformed into the strains via electroporation, and the strains comprising the plasmids integrated into the chromosome were selected for on M17 plates supplemented with glucose and erythromycin. Afterwards, the transformants were purified and plated on SA glucose medium (Jensen et al., 1993) plates supplemented with 5-fluoroorotate, thereby selecting for strains in which the plasmid had been lost by homologous recombination. The successful deletions were verified by PCR (Solem et al., 2008).

1.4 Deleting genes from the *Lactococcus lactis* subsp. *cremoris*

The following genes were deleted from the *Lactococcus lactis* subsp. *cremoris* parent strain *ldhX, ldhB, ldh, pta,* and *adhE*. The genes were deleted using gene deletion plasmids derived from pCS1966 designated as: pCS4026 (*ldhX*), pCS4020 (*ldhB*), pCS4104 (*ldh*), pCS4230 (*pta*), pCS4273 (*adhE*), constructed as described above (Example 1.2).

Deletion of the genes from the *Lactococcus lactis* subsp. *cremoris* parent strain was verified by PCR amplification of the respective gene using primers 774/777 (*ldhX*), 769/771 (*ldhB*), 788/789 (*ldh*), 880/881(*pta*) and 929/930 (*adhE*).

The strain containing the three lactate dehydrogenase deletions (*ldh, ldhB, ldhX*) was named CS4099 or MG1363Δ3ldh. CS4234 (MG1363 Δ^3*ldhΔpta*) was derived from CS4099, by additionally the deleting a phosphotransacetylase gene, *pta* using pCS4230. CS4363 (MG1363 Δ^3*ldhΔptaΔadhE*) was derived from CS4234, by additionally the deleting the native adhE gene using pCS4273, using the gene deletion methods described in section 1.3.

Table 1 Strains and plasmids

Designation	Genotype or description	Reference

***L. lactis* strains**		
MG1363	Wild type	Gasson,1983
CS4099	MG1363 *Δ3ldh (ΔldhB, ΔldhB & ΔldhX)*	Solem et al., 2013
CS4234	MG1363 *Δ3ldh Δpta*	-"-
CS4363	MG1363 *Δ^{3}ldh Δpta ΔadhE*	-"-
CS4435	MG1363 *Δ^{3}ldh Δpta ΔadhE* pCS4268	-"-
CS4435L	MG1363 *Δ^{3}ldh Δpta ΔadhE* pCS4268 pLP712	This work
pG$^+$host8	*E. coli*/*L. lactis* shuttle vector, Tet^R, thermosensitive replicon	Maguin et al., 1996
pCS4268	pG$^+$host8::SP-*ldh* (*L. lactis*)	This work
pCS4564	pG$^+$host8::SP-*ldhA* (*E. coli*)	This work
pCI372	*E. coli*/*L. lactis* shuttle vector, Cam^R	Hayes et al., 1990
pCS4518	pCI372::*gusA*	This work
pLP712	Lac plasmid from NCDO712	Wegmann et al., 2002

Abbreviations: *ldh* (*ldhB, ldhX*), lactate dehydrogenase genes; *pta*, phosphotransacetylase gene; *adhE*: alcohol dehydrogenase genes; pCS4268: pTD6-*pdc-adhB* (*pdc*, pyruvate decarboxylase gene from *Zymomonas mobilis*; *adhB*, ethanol dehydrogenase gene from *Z. mobilis*).

1.5 Introducing codon-optimized genes encoding pyruvate decarboxylase and alcohol dehydrogenase B into *L. lactis* strain MG1363 *Δ^{3}ldhΔptaΔadhE*

The pyruvate decarboxylase gene (*pdc*) was amplified using primers 690/829 and the alcohol dehydrogenase gene (*adhB*) was amplified using 830/791. In both cases the templates were synthetic codon-optimized genes based on *Zymomonas mobilis pdc* and *adhB* genes (GenScript). An SP (synthetic promoter)-*pdc-adhB* fragment was amplified from CS4116 chromosomal DNA using primers 947/894, phosphorylated using T4 polynucleotide kinase and ligated to pTD6 amplified using 891/892. The ligation was transformed directly into CS4363 (MG1363 *Δ3ldhΔptaΔadhE*). CS4435 is a strain comprising the plasmid pCS4268 comprising and expressing the *pdc-adhB* genes. The plasmid pTD6 is a derivative of pAK80 (Solem et al., 2013) containing a *gusA* reporter gene. A PCR fragment was amplified from pAK80 using primers ptd45/ptd46. The gusA gene was amplified from *E. coli* MG1655 using primers ptd1/ptd2. After digesting both fragments with *Bgl*II and *Avr*II the fragments were ligated and transformed into *E. coli* strain MC1000. The resulting plasmid was named pTD5. The erythromycin marker of pTD5 was replaced by a tetracycline marker by PCR amplifying pTD5 using primers ptd49/ptd50, digesting the product with *Stu*I/*Apa*I and ligating it with a *Stu*I/*Apa*I fragment containing the tetracycline resistance genes amplified

from pG+host8 (29) using primers ptd51/ptd52. The ligation was introduced in *E. coli* MC1000 and the result was plasmid pTD6.

Table 2 Primers

Primer name	Primer use	Primer sequence (5' → 3')	SEQ ID No.
43 (T3)	Verify insert in pCS1966	AATTAACCCTCACTAAAGGG	99
263	5' end of *gusA*, rev.	CGCGATCCAGACTGAATG	100
603	Verify insert in pCS1966	ATCAACCTTTGATACAAGGTTG	101
690	Modulate *pdc* expression, XbaI	CTAGTCTAGACTGAACCCTACCGNNNNNAGTTTATT CTTGACANNNNNNNNNNNNNNTGRTATAATNNNNA AGTAATAAAATATTCGGAGGAATTTTGAAATGAGTT ATACAGTAGGAAC	102
691	Used with 690, PstI	TCGGACTGCAGTTACAACAATTTATTTACAGGTTTTC	103
702	pLB85	GAGTCGACCTGCAGAATTC	104
703	pLB85, XbaI	TCGACTCTAGAGGATCTAC	105
768	*ldhB* ups., PstI	AATTCCTGCAGCATATTAAATAATGAACAAGTCATT C	106
769	*ldhB* ups., BamHI	TAGTGGATCCTGGTAAATCCAAACACAACAAC	107
770	*ldhB* dwn., PstI	AATTCCTGCAGTAATTTCCAGCTCTTACAATAAC	108
771	*ldhB* dwn., XhoI	GACCTCGAGTCAGAAACTTTCTTTACCAGAG	109
772	pCS1966, BamHI	GCGGGGATCCACTAGTTCTAG	110
773	pCS1966, XhoI	ATACCGTCGACCTCGAG	111
774	*ldhX* ups., BamHI	TAGTGGATCCCTGTTTCAGGTCTTGGATAG	112
775	*ldhX* ups., EcoRI	CCGATGAATTCTCATTAGCACGTTTAACAAGAG	113
776	*ldhX* dwn., EcoRI	CCGATGAATTCATCAGCGTAGTCTGCTGC	114
777	*ldhX* dwn., KpnI	CGGGGTACCATTTAATCCTAAAGTCGTTATTAC	115
785	*ldh* ups., EcoRI	CCGATGAATTCTTAAGTCAAGACAACGAGGTC	116
786	*ldh* dwn, EcoRI	CCGATGAATTCGACCTTGTTGAAAAAAATCTTC	117
787	*ldh* ups., BamHI	TAGTGGATCCGTACAATGGCTACTGTTAAC	118
788	*ldh* dwn., XhoI	GACCTCGAGGATGAACAGACTTTTTTATTATAG	119
789	Verify *ldh* deletion	AAAACCAGGTGAAACTCGTC	120
791	*adhB* rev, PstI	TCGGACTGCAGTTAAAATGCTGATAAAAACAATTCT TC	121
827	pCS1966, BamHI	ATACCGTCGACCTCGAG	122
828	pCS1966, XhoI	CGATAAGCTTGATATCGAATTC	123
829	Used with 690, EcoRI	CCGATGAATTCTTACAACAATTTATTTACAGGTTTTC	124
830	*adhB* fwd, EcoRI	CCGATGAATTCTATAAGGAGAATTAGAATGGCAAGT AGTACATTTT ATATTC	125
834	*pfl* ups., XbaI	CTAGTCTAGACAAGTGATGTACCAAATGAC	126
835	*pfl* ups., BamHI	CGCGGATCCTTTGAAATCTCCTTTGTTCT	127
836	*pfl* dwn., BamHI	CGCGGATCCTTCTTAGTATTAAAAAATATAAAG	128
837	*pfl* dwn., XhoI	GGTACTCGAGTGTGATTCACCCCTATTTCT	129
845	Synthetic promoter primer, *pdc*, XbaI	CTAGTCTAGACTGAACCCTACCGTGGGGAGTTTATT CTTGACAAGTTCTCTTGGAGTTGATATAATCAAGAA GTAATAAAATATTCGGAGGAATTTTG	130
846	Synthetic promoter primer, *pdc*, XbaI	CTAGTCTAGACTGAACCCTACCGGTCAGAGTTTATT CTTGACATCGTTACCGTAGGTTGGTATAATCTTGAA GTAATAAAATATTCGGAGGAATTTTG	131
847	Synthetic promoter primer, *pdc*, XbaI	CTAGTCTAGACTGAACCCTACCGGCAAGAGTTTATT CTTGACAATTACCTGTTCGCTTGATATAATAAGGAA GTAATAAAATATTCGGAGGAATTTTG	132
848	Synthetic promoter primer, *pdc*, XbaI	CTAGTCTAGACTGAACCCTACCGGTCGAAGTTTATT CTTGACAATCTGTGAGATCAGTGATATAATACAGAA	133

		GTAATAAAATATTCGGAGGAATTTTG	
878	*pta* ups., USER	ATCCCTCGGTTACAAGTTTCU	134
879	*pta* dwn., USER	AGAAACTTGTAACCGAGGGAUAATAATAGATTGAA ATTCTGTCAG	135
880	*pta* ups., USER	ATTCGATATCAAGCTTATCGAUCAAAAATTGTGGTA GAATATATAG	136
881	*pta* dwn., USER	AGGTCGACGGTATCGATAAUCCTAGTTCAATTGATG TGAC	137
882	pCS1966, USER	ATCGATAAGCTTGATATCGAAU	138
883	pCS1966, USER	ATTATCGATACCGTCGACCU	139
891	pTD6, USER	ACAGATTAAAGGTTGACCAGTAU	140
892	pTD6, USER	ACCAATTCTGTGTTGCGCAU	141
894	*adhB* rev., USER	ATACTGGTCAACCTTTAATCTGUTTAAAATGCTGAT AAAAACAATTCTT	142
920	pCS1966, USER	ATAAGCTUGATATCGAATTCCT	143
921	pCS1966, USER	ATTCCCTTUAGTGAGGGTTAAT	144
927	*adhE* ups., USER	ATGTGTACGUTCTCCTTTGTG	145
928	*adhE* dwn., USER	ACGTACACAUATTATAGTATTTGGAACCGAAC	146
929	*adhE* ups., USER	AAGCTTAUGGTCGTCTTGTTACTTGTG	147
930	*adhE* dwn., USER	AAAGGGAAUTCTGCCGGAGCTATATATG	148
947	Modulate *pdc* expression, USER	ATGCGCAACACAGAATTGGUGGCCNNNNNAGTTTA TTCYYRAMANNNNNNNNNNNNNNTGRYAYAAYNN NNAAGTAATAAAATATTCGGAGGAAT	149
ptd1	*gusA* fwd.	GACTGAACTAGTAGATCTGCAGAAGCTTGTCGACCC GGGTACCTCGAGCTCCATGGCATATGCGGCCGCATG CGCAACACAGAATTGGTTAAC	150
ptd2	*gusA* rev.	ACCTCTCCTAGGATTAAAAAATAAAAAAGAACCCA CTCGGGTTCTTTTTTTTACTAGCTAGCTAATGGTGCG CCAGGAGAGTTG	151
ptd45	pAK80	TTCTGCAGATCTTCAGTCAGTCAAGAGGTTTGATGA CTTTGAC	152
ptd46	pAK80	TTTAATCCTAGGGATTGTGGGAAATTTAGGCG	153
ptd49	pTD5, StuI	ATGCTAGGGCCCACGGGGAATTTGTATCGATG	154
ptd50	pTD5, ApaI	TGATAAAGGCCTTACTGCACTATCAACACACT	155
ptd51	pG^+host8, StuI	TTAAGAAGGCCTAAGAAATTTCGCCAGTCG	156
Ptd52	pG^+host8, ApaI	GCGCCTGGGCCCGCCACTCATAGTTCTAAAC	157

1.6 Introducing genes encoding the lactose catabolism pathway into *Lactococcus lactis* subsp. *cremoris* strain MG1363 Δ^3*ldhΔptaΔadhE*

The wild type strain *L. lactis* MG1363, and its derivatives described herein (e.g. CS4435), are plasmid-free strains that cannot utilize lactose as a carbon source. The *Lactococcus* plasmid, pLP712 (55.395 kbp), comprises genes encoding the entire lactose catabolism pathway (Wegmann et al., 2012). The lactose-metabolism plasmid pLP712 (55,395 bp) was extracted from the dairy isolate NCDO712 based on the method of Andersen (1983); and then transformed into *L. lactis* strain CS4435 to give strain CS4435L.

The lactose catabolism pathway genes located on the pLP712 plasmid (Figure 2) are as follows:

1. the *lacAB* genes encoding two subunit polypeptides that together have galactose-6-phosphate isomerase activity (EC 5.3.1.26); wherein the first subunit polypeptide has an amino acid sequence of SEQ ID NO: 90 encoded by the *L. lactis lacA* gene; and the second subunit polypeptide has an amino acid sequence of SEQ ID NO: 92 encoded by the *L. lactis lacB* gene;

2. the *lacC* gene encoding a polypeptide having D-tagatose-6-phosphate kinase activity (EC 2.7.1.114); wherein the polypeptide has an amino acid sequence of SEQ ID NO: 94);

3. the *lacD* gene encoding a polypeptide having tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40); wherein the polypeptide has an amino acid of SEQ ID NO: 96;

4. the *lacEF* genes encoding a two polypeptide components together having lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69); wherein the first polypeptide component is a phosphotransferase system EIICB component having an amino acid sequence of SEQ ID NO: 84, encoded by the *L. lactis lacE* gene; and the second polypeptide component is a phosphotransferase system EIIA component having an amino acid sequence of SEQ ID NO: 86, encoded by the *L. lactis lacF* gene; and

5. the *lacG* gene encoding a polypeptide having phospho-β-D-galactosidase activity (EC 3.2.1.85); wherein the polypeptide has the amino acid sequence of the phospho-β-D-galactosidase of SEQ ID NO: 88; and

6. the lacR gene encoding a lactose transport regulator of SEQ ID NO: 98

1.7 Fermentation properties of the lactic acid bacterium genetically modified for ethanol production

The wild type strain MG1363 and its genetically modified derivative strains, as listed in Table 3, were cultivated in defined SA medium (Jensen et al., 1993) with glucose and samples of each culture were collected after 24 hours. Cell growth was regularly measured by OD^{600} and quantification of lactose, glucose, lactate, formate, acetate, ethanol, acetoin and 2,3-butanediol was carried out using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with a Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60°C and the mobile phase of consisted of 5 mM H_2SO_4, at a flow rate of 0.5 ml/min. CO_2 is

included for calculating the carbon balance. Values are averages of three independent experiments and standard deviations are indicated.

Lactococcus lactis is characterised by homolactic fermentation when grown on glucose, as illustrated for the wild-type strain MG1363 in Table 3. Following deletion of the three *ldh* genes, in strain CS4099, the dominant products become acetate and ethanol, while the production of acetate was eliminated by deleting the *pta* gene, in strain CS4234, however this strain still produced significant amounts of formate and acetoin, and small amounts of 2,3-butandiol . The additional deletion of the native *adhE* gene yielded strain CS4363 that was unable to grow under anaerobic conditions because of the defect in cofactor regeneration ability. However, its growth could be restored in the presence of O_2, where NAD^+ is recycled by NADH oxidase (NoxE) which results in acetoin as the main fermentation product. The introduction of *pdc* and *adhB* genes, however, creating strain CS4435, restored complete cofactor-recycling and bacterial growth under anaerobic conditions, and the outcome was a genetically modified *L. lactis* strain capable of homo-ethanol production from glucose as carbon source (Table 3). The native *adhE* gene encodes a bifunctional ADHE that can use both acetyl-CoA and acetaldehyde as substrate, while the transgene *adhB*, encoding an ADHB derived from from *Z. mobilis* only uses acetaldehyde. As a consequence, the cofactors are completely balanced in the heterologous ethanol forming pathway and nearly all the carbon balance is converted to ethanol.

Table 3 Fermentation products of wild type and genetically modified *L. lactis*

Strains	Carbon balance	Lactate	Acetate	Formate	Acetoin	2,3-Butanediol	Ethanol
		mol/mol glucose					
MG1363	90%	**1.8 ± 0.1**	ND	ND	ND	ND	**ND**
CS4099	85%	**ND**	0.61 ± 0.02	1.58± 0.07	0.08 ± 0.01	0.09± 0.01	**0.65 ± 0.02**
CS4234	88%	**ND**	ND	0.82± 0.05	0.35± 0.02	0.16± 0.03	**0.76 ± 0.04**
CS4363	85%	**ND**	ND	ND	0.85± 0.05	ND	**ND**
CS4435	87%	**ND**	ND	ND	ND	ND	**1.75 ± 0.06**

The strain CS4435L, derived from strain CS4435, comprises the *Lactococcal* plasmid, pLP712 (55.395 kbp) that encodes the entire lactose catabolism

pathway (figure 2). Strain CS4435L was grown on a defined SA medium (Jensen et al., 1993) comprising 7.2 g/L lactose as the sole carbon source. As seen in figure 3, the cells grew and consumed the supplied lactose completely within 11 hours and the only fermentation product was ethanol. The measured growth rate was 0.6 h^{-1}, close to the growth rate on glucose (0.7 h^{-1}). The ethanol concentration reached during cultivation of strain CS4435L was 3.2 g/L, with a yield of 0.45 g ethanol/g lactose, corresponding to 83% of the theoretical maximum. The final ethanol titer was increased to 6.7, 10.3 and 12.0 g/L when the initial lactose concentration was increased to 15.1, 24.0 and 31.8 g/L, respectively (Table 2).

Table 4 Optimization of lactose concentrations in different types of media.

Lactose (g/L)	Media types	OD_{600}	Ethanol (g/L)	Yield (g ethanol/g lactose)	Conversion efficiency[a]
7.2	SA[b]	2.0	3.2	0.45	0.83
15.1	SA[b]	2.3	6.7	0.44	0.82
24.0	SA[b]	2.9	10.3	0.43	0.80
31.8	SA[b]	3.6	12.0	0.38	0.71
32.0	RWP+YE[c]	7.2	13.4	0.42	0.78
40.0	RWP+CSLH[d]	4.0	17.5	0.44	0.82
80.0	RWP+CSLH[e]	6.0	30.6	0.40	0.71
80.0[m]	RWP+CSLH[f]	6.0	41.0	0.38	0.70

a = Conversion efficiency was calculated based on the theoretical maximal yield (0.538 ethanol/g lactose).
b = The composition of SA medium (Jensen et al. 1993) comprises 19 amino acids, vitamins and salts. Glucose was replaced by lactose.
c = Diluted RWP (residual whey permeate) and 0.5% (w/v) YE (yeast extract).
d-f = Diluted RWP (residual whey permeate) and 2.5% (w/v) CSLH (corn steep liquor hydrolysate), prepared by condition H1.
m = Fed-batch was performed with initial 80 g/L lactose and the details can be found in Fig. 7.

Example 2 Development of a low cost medium for production of ethanol from lactose by genetically modified *Lactococcus lactis* strain of the invention

Waste stream residual whey permeate (RWP) is the permeate mother liquor after extracting lactose from whey permeate. The composition of the RWP, which was supplied by Arla Foods Ingredients Group P/S (http://www.arlafoodsingredients.com) was determined and shown in Table 5. The sugar components of a filtered sample of RWP were determined as described in example 1.6, and the amino acid composition was determined by

the steps of hydrolysis of the filtered sample with 6 M HCl; separation by ion exchange chromatography and detection after oxidation and derivatization with o-phthaldialdehyde, as described by Barkholt et al., (1989).

Table 5 The composition of residual whey permeate[a]

Composition	Concentration
Lactose	150 g/L
Galactose	3 g/L
Aspartate	0.252 mM (mmol/L)
Threonine	0.076 mM
Serine	0.088 mM
Glutamate	1.464 mM
Proline	0.384 mM
Glycine	0.904 mM
Alanine	0.24 mM
Cysteine	0.096 mM
Valine	0.072 mM
Methionine	0.124 mM
isoleucine	0.04 mM
Leucine	0.092 mM
Histidine	0.208 mM
Lysine	0.304 mM
Arginine	0.096 mM

[a] Residual whey permeate is a concentrate of the residue remaining after lactose extraction from whey permeate.

Although strain CS4435L both grew and produced ethanol when cultured on defined SA medium (Jensen et al., 1993) comprising 7.2 g/L lactose; the strain was unable to grow or produce ethanol formation when cultured on RWP alone, even though an initial the lactose content of 50 g/L lactose was provided (Figure 4A-B). In view of the relatively low amino acid content of RWP, various nitrogen supplements, added at different concentrations to the RWP, were tested to support growth and ethanol production of strain CS4435L. As seen in figure 4, CS4435L was unable to grow in RWP supplemented with inorganic NH_4Cl. However, the addition of yeast extract (YE) enhanced growth; and after 12 hours fermentation on a RWP medium comprising 0.5% (w/v) YE, the culture reached a final cell density ($OD600_{nm}$) of 6.5, and a final ethanol concentration was nearly 19 g/L. Accordingly, growth and ethanol production CS4435L on a RWP medium requires the addition of a complex nitrogen source.

Since the price of YE is too high to provide the basis for a low cost medium; corn steep liquor (CSL) was tested as a cheap source of complex nitrogen. CSL was purchased from Sigma–Aldrich (St. Louis, MO) with 40-60% solid content. RWP medium supplemented with CSL, at concentrations ranging from 0.1% to 2.5% (w/v), however, only supported low levels of cell growth of strain CS4435L, and levels of ethanol produced were also very low. A combination of 0.1% (w/v) YE with CSL, led to only a small stimulation of growth (Figure 4A-B).

In order to enhance the available amino nitrogen content of CSL, samples of CSL were subjected to various degrees of acid hydrolysis. The acid hydrolysis was performed with very small amounts of sulfuric acid (0.05-0.5% concentrated H_2SO_4 added to CSL having a 20-30% w/v solid content). The following hydrolysis conditions were applied to produce corn steep liquor hydrolysates (CSLH). H1 condition: original CSL was diluted 2 times with water and then 50 µl concentrated sulfuric acid was mixed with 100 ml diluted CSL. The mixture was kept at 121°C for 15 mins and subsequently the pH was adjusted to 6.8-7.1 with the addition of 10 M NaOH solution. H2 condition: original CSL was diluted 2 times with water and then 250 µl concentrated sulfuric acid was mixed with 100 ml diluted CSL. The mixture was kept at 121°C for 15 mins and subsequently the pH was adjusted to 6.8-7.1 with the addition of 10 M NaOH solution. H3 condition: original CSL was diluted 2 times with water and then 500 µl concentrated sulfuric acid was mixed with 100 ml diluted CSL. The mixture was kept at 121°C for 15 mins and subsequently pH was adjusted to 6.8-7.1 with the addition of 10 M NaOH solution.

When comparing the growth and ethanol production of CS4435L cultured on RWP supplemented with the CSL hydrolysates (CSLH), the use of H1 hydrolysate (CSL treated with 0.05% H_2SO_4) gave the greatest increase in production levels (Figure 4C-D). Increasing the amount of sulfuric acid used during acid hydrolysis (H2, 0.25% H_2SO_4) did not further improve the ability of the CSLH to support cell growth and ethanol production, and even had a negative effect at the highest acid concentration tested (H3, 0.5% H_2SO_4).

Thus, a cell density of only 3.0 ($OD600_{nm}$) and the final ethanol titer of only 11 g/L was obtained when using 2.5% (w/v) CSLH.

Analysis of the free amino acid composition of CSL revealed that hydrolysis of corn steep liquor increases the free amino acid content of CSL by circa 2 fold in comparison with untreated corn steep liquor.

Table 6 Free amino acid composition of CSL before and after hydrolysis

Amino acids Unit (**mM**)	CSL 25 % (w/v)	Hydrolyzed CSL (H1) 25 % (w/v)
Aspartate	1.8	3.6
Glutamate	0.9	2
Asparagine	1.4	3
Glutamine	0.8	2.1
Histidine	6.1	11
Arginine	4.5	10.5
Alanine	2.1	5
Tyrosine	0.8	2.1
Cysteine	3.7	5.7
Valine	2.1	4.8
Isoleucine	1.8	3.6
Leucine	0.8	2.5
Methionine	0.1	1.2

A direct correlation was observed between the amount of CSLH added to the RWP medium and the final cell biomass and ethanol produced (figure 4C-D). When the RWP was supplemented with 2.5% (w/v) corn steep liquor hydrolysate (CSLH), a high final cell density of 4.5 ($OD600_{nm}$) and a high ethanol titer (17.5 g/L) were obtained after 30 hours of fermentation from 50 g/L lactose. The ethanol yield obtained with CS4435L was only marginally less than that achieved when cells were grown on RWP supplemented with 0.5% (w/v) YE; although cell growth was reduced compared to the YE supplemented media.

The composition of two tested low cost growth media composed of RWP supplemented with hydrolysed CSL is shown in Table 7.

Table 7 Composition of two initial growth media comprising

	Low lactose Medium 1		High lactose Medium 2	
Composition	RWP 50g/L lactose	CSLH (H1) 2.5% w/v	RWP 80g/L lactose	CSLH (H1) 5% w/v
Lactose (g/L)	50 g/L		80 g/L	
Galactose (g/L)	1 g/L		1.6 g/L	
Aspartate (mM)	0.08	0.36	0.135	0.72
Threonine (mM)	0.025		0.040	
Serine (mM)	0.003		0.047	
Glutamate (mM)	0.488	0.20	0.787	0.40
Proline (mM)	0.012		0.206	
Glycine (mM)	0.301		0.480	
Alanine (mM)	0.08	0.50	0.129	1.00
Cysteine (mM)	0.032	0.57	0.051	1.10
Valine (mM)	0.024	0.48	0.038	0.96
Methionine (mM)	0.041	0.12	0.066	0.24
Isoleucine (mM)	0.04	0.36	0.021	0.72
Leucine (mM)	0.092	0.25	0.049	0.50
Histidine (mM)	0.069	1.10	0.112	2.20
Lysine (mM)	0.101		0.163	
Arginine (mM)	0.032	0.30	0.051	0.60
Glutamine (mM)		0.20		0.40
Tyrosine (mM)		0.21		0.42

The low cost growth medium composed of RWP, used in an amount conferring a lactose concentration of either 50 g/L or 80 g/L when supplemented with 2.5% or 5% CSLH (H1) respectively, provides lactose as substrate for ethanol production and amino acids in sufficient amounts to support ethanol production and growth. The 2.5% or 5% CSLH, hydrolysed under the conditions of H1 is sufficient to enhance the levels of available complex amino nitrogen (soluble polypeptides, peptides and free amino acids), and in particular the levels of the essential amino acids glutamine, histidine, methionine, leucine, isoleucine, and valine.

Example 3 Optimizing ethanol production by genetically modified *Lactococcus lactis* strain of the invention on a low cost medium

Ethanol production by *L. lactis* strain CS4435L and cell growth during fermentation on the low cost medium, RWP supplemented with 2.5% (w/v)

CSLH (H1), was monitored in order to determine the yields obtainable by this process. Cell growth reached a final cell density ($OD600_{nm}$) of 4.0 after 14.5 hours in medium with 2.5% (w/v) CSLH (H1); and the initial lactose content of 40 g/L lactose was completely consumed within 31 hours (figure 5B). The ethanol concentration increased linearly to 17.5 g/L, corresponding to 82% of the theoretical maximum (Table 4). By comparison, fermentation RWP supplemented with 0.5% (w/v) YE (figure 5A), gave a lower ethanol yield of 13.4 g/L, corresponding to 78% of the theoretical maximum (Table 4), while cell growth was faster and the final cell density was higher ($OD600_{nm}$=7.2). Thus, the replacement of YE with cheap CSLH as a supplement to the RWP medium resulted in a 44% decrease in cell biomass yield, but an overall increase in ethanol production yield.

Enhanced ethanol titers obtainable with *L. lactis* strain CS4435L, during fermentation on the low cost medium, RWP supplemented with 2.5% (w/v) CSLH (H1), were achieved by raising the initial lactose content of the medium. When the initial lactose content of the medium was raised to 80 g/L lactose, the lactose was totally consumed after 55 hours fermentation (figure 6), where the ethanol titer reached 30.6 g/L, corresponding to a yield of 71% (Table 4). However, even higher initial lactose content of the medium provided at the beginning of the fermentation failed to further enhance the ethanol titer.

In order to enhance lactose supply during fermentation, without further elevating the lactose concentration in the low cost medium, fed-batch fermentation method was implemented, whereby lactose was added during fermentation. Specifically, fed-batch was performed with diluted RWP comprising a lactose content of 80 g/L lactose supplemented with 2.5% (w/v) CSLH; and 500 g/L lactose stock solution was used for feeding. The feeding was performed when the lactose concentration was lower than 10 g/L and after rapid injection it returned to around 20 g/L.

Using this fermentation strategy using *L. lactis* strain CS4435L on the low cost medium (figure 7), gave a final titer of 41 g/L ethanol after nearly 90 hours,

corresponding to a yield of 70% (Table 4). The achieved ethanol titer of 5.2% meets the requirements for subsequent low cost ethanol distillation.

Example 4 Treatment of CSL as a source of amino nitrogen for a genetically modified *Lactococcus* lactis strain

In order for *L. lactis* strain CS4435L to grow and produce ethanol when cultured on diluted residual whey permeate medium and 80 g/L lactose; it requires a source of amino nitrogen (Example 2). Example 2 further shows that CSL can only serve as a cheap source of amino nitrogen when it is hydrolysed. The present example compares three different methods of hydrolysing CSL included in the growth medium for *L. lactis* strain CS4435L, and their respective impacts on ethanol production. The CSl was either hydrolysed with acid or it was proteolytically hydrolysed. Figure 8 shows ethanol production over time by a *L. lactis* strain CS4435L when cultured on diluted residual whey permeate medium and 80 g/L lactose supplemented with one of: (1) 2.5% (w/v) CSLH prepared under H1 conditions (CSL treated with 0.05% H_2SO_4 as described in Example 2); (2) 2.5% (w/v) CSL and 70 IU/L proteinase; and (3) 2.5% (w/v) CSL and 700 IU/L proteinase (where the protease was added to the growth medium prior to cultivation. Samples were collected periodically for determining ethanol concentration (filled triangles), over a period of 40 -50 hours as displayed. The proteinase was derived from *Aspergillus melleus* Type XXIII (P4032 supplied by Sigma).

Surprisingly, growth media comprising acid hydrolysed CSLH supported significantly higher levels of ethanol production than proteinase-treated CSL. Thus, *L. lactis* strain CS4435L cultures grown in media supplemented with CSLH produced 24 g/L ethanol in 39 h; while cultures grown in media supplemented with 70 IU/L or 700 IU/L proteinase-treated CSL, only produced 8.9 g/L andr 10.7 g/L ethanol in 48 h, respectively.

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important composite metabolic plasmid pLP712. Microbiol., 158:2936–2945

Claims

1. A method for ethanol production using a genetically engineered lactic acid bacterium comprising the steps of:
   a. introducing a genetically modified lactic acid bacterium into an aqueous culture medium;
   b. incubating the culture of (a);
   c. recovering ethanol produced by said culture during step (b), and optionally
   d. isolating the recovered ethanol;
   wherein the aqueous culture medium comprises:
   I. whey permeate or residual whey permeate, and
   II. an amino nitrogen source, and
   wherein the genetically engineered lactic acid bacterium comprises transgenes encoding:
   i. a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1); and
   ii. a polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1); and
   wherein the genome of said lactic acid bacterium comprises genes encoding polypeptides having:
   iii. lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69)
   iv. phospho-β-D-galactosidase activity (EC 3.2.1.85)
   v. galactose-6-phosphate isomerase activity (EC 5.3.1.26),
   vi. D-tagatose-6-phosphate kinase activity (EC 2.7.1.114), and
   vii. tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40);
   wherein the genome of said lactic acid bacterium is deleted for genes or lacks functional genes or genes encoding polypeptides having an enzymatic activity of:
   viii. lactate dehydrogenase (E.C 1.1.1.27 or E.C.1.1.1.28)
   ix. phosphotransacetylase (E.C.2.3.1.8) and

x. bifunctional alcohol dehydrogenase (E.C. 1.1.1.1 and EC 1.2.1.10).

2. A method for ethanol production according to claim 1, wherein the amino nitrogen source is acid hydrolysed corn steep liquor (CSLH) wherein the concentration of at least one free amino acid, selected from the group consisting on glutamine, histidine, methionine, leucine, isoleucine, and valine is at least 1.5 fold greater than the concentration of the corresponding amino acid in the original corn steep liquor from which the CSLH was derived.

3. A method for ethanol production according to claim 1 or 2, wherein the lactose content of the medium at step a) is from 20 g to 200 g lactose/L.

4. A method for ethanol production according to claim 2 or 3 , wherein the CSLH w/v solids content of the medium at step a) is from 2% to 20%.

5. A method for ethanol production according to any one of claims 2 to 4, wherein the content of free histidine provided by the CSLH in the aqueous culture medium is at least 0.8mM.

6. A method for ethanol production according to any one of claims 2 to 5, wherein the aqueous culture medium further comprises yeast extract.

7. A method for ethanol production according to any one of claims 1 to 6, wherein the culture is fed-batch; and wherein the culture in step b) is fed with at least lactose.

8. A method for ethanol production according to any one of claims 2 to 7, wherein the aqueous culture medium consists of the components: the residual whey permeate; the CSLH; water and optionally supplemented with yeast extract and/or an aqueous solution of lactose.

9. A method for ethanol production according to any one of claims 1 to 8, wherein the lactic acid bacteria belongs to a genus selected from the group consisting of *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc, Streptococcus, Oenococcus*, and *Bacillus*.

10. A method for ethanol production according to any one of claims 1 to 9, wherein:
    a. the polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1) has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 80; and
    b. the polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1) has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 82.

11. A method for ethanol production according to any one of claims 1 to 10, wherein:
    a. the amino acid sequence of the polypeptide having lactate dehydrogenase activity has at least 80% sequence identity to an amino acid sequence selected from among SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34 and 36;
    b. the amino acid sequence of the polypeptide having phosphotransacetylase activity has at least 80% sequence identity to an amino acid sequence selected from among SEQ ID NO: 42, 44, 46, 48, 50, 52, 54, 56, 58 and 60;
    c. the amino acid sequence of the polypeptide having bifunctional alcohol dehydrogenase activity has at least 80% sequence identity to an amino acid sequence selected from among SEQ ID NO: 62, 64, 66, 68, 70, 72, 74, 76 and 78.

12. A method for ethanol production according to any one of claims 1 to 11, wherein:
    a. the lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69) is provided by a first and a second polypeptide, wherein the amino acid sequence of the first polypeptide has at least 80% sequence identity to an amino acid sequence of SEQ

ID NO: 84, and the amino acid sequence of the second polypeptide has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 86;

b. the amino acid sequence of the polypeptide having phospho-β-D-galactosidase activity (EC 3.2.1.85) has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 88;

c. the amino acid sequence of the polypeptide having galactose-6-phosphate isomerase activity (EC 5.3.1.26) is provided by a first and a second polypeptide, wherein the amino acid sequence of the first polypeptide has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 90 and the amino acid sequence of the second polypeptide has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 92;

d. the amino acid sequence of the polypeptide having D-tagatose-6-phosphate kinase activity (EC 2.7.1.114) has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 94; and

e. the amino acid sequence of the polypeptide having tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40) has at least 80% sequence identity to an amino acid sequence of SEQ ID NO: 96.

13. Use of a genetically engineered lactic acid bacterium for the production of ethanol from an aqueous culture medium comprising
I. whey premeate or residual whey permeate, and
II. an amino nitrogen source;
wherein the genetically engineered lactic acid bacterium comprises transgenes encoding:

i. a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1); and

ii. a polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1); and

wherein the genome of said lactic acid bacterium comprises genes encoding polypeptides having:

iii. lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69)

iv. phospho-β-D-galactosidase activity (EC 3.2.1.85)

v. galactose-6-phosphate isomerase activity (EC 5.3.1.26),

vi. D-tagatose-6-phosphate kinase activity (EC 2.7.1.114), and

vii. tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40); and

wherein the genome of said lactic acid bacterium is deleted for genes or lacks functional genes encoding polypeptides having an enzymatic activity of:

viii. lactate dehydrogenase (E.C 1.1.1.27 or E.C.1.1.1.28)

ix. phosphotransacetylase (E.C.2.3.1.8) and

x. bifunctional alcohol dehydrogenase (E.C. 1.1.1.1 and EC 1.2.1.10).

14. The use of a genetically engineered lactic acid bacterium for the production of ethanol according to claim 13, wherein the amino nitrogen source is acid hydrolysed corn steep liquor (CSLH), wherein the concentration of at least one free amino acid, selected from the group consisting on glutamine, histidine, methionine, leucine, isoleucine, and valine is at least 1.5 fold greater than the concentration of the corresponding amino acid in the original corn steep liquor from which the CSLH was derived.

15. A genetically engineered lactic acid bacterium for the production of ethanol from an aqueous culture medium comprising whey permeate or residual whey permeate, and an amino nitrogen source; wherein the genetically engineered lactic acid bacterium comprises transgenes encoding:

i. a polypeptide having pyruvate decarboxylase (PDC) activity (EC 4.1.1.1); and

ii. a polypeptide having alcohol dehydrogenase B activity (EC 1.1.1.1); and

wherein the genome of said lactic acid bacterium comprises genes encoding polypeptides having:

iii. lactose-specific phosphotransferase system (PTS) activity (EC 2.7.1.69)

iv. phospho-β-D-galactosidase activity (EC 3.2.1.85)

v. galactose-6-phosphate isomerase activity (EC 5.3.1.26),

vi. D-tagatose-6-phosphate kinase activity (EC 2.7.1.114), and

vii. tagatose 1,6-diphosphate aldolase activity (EC 4.1.2.40); and

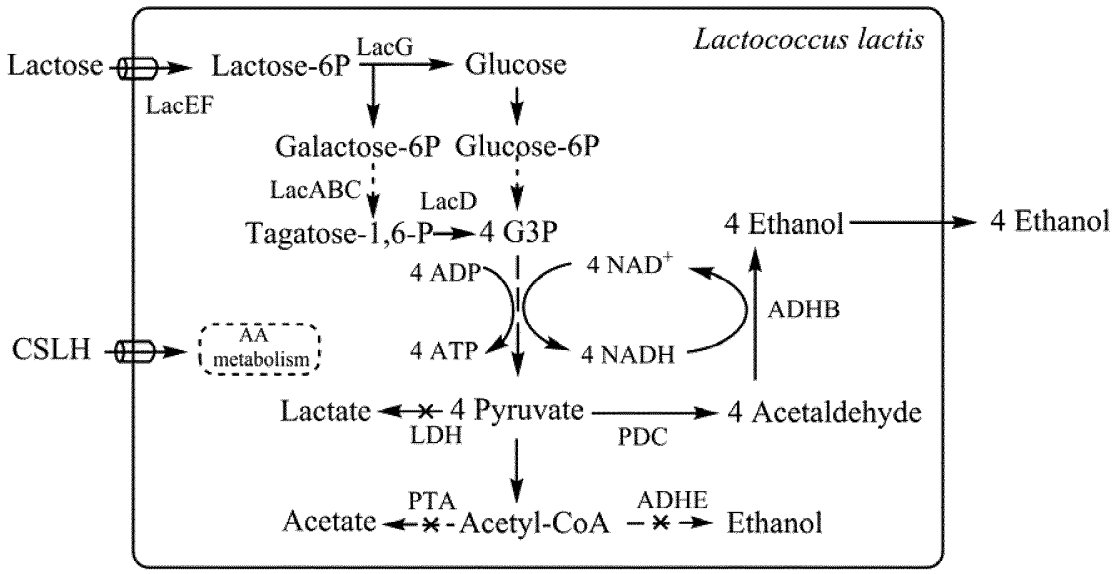
wherein the genome of said lactic acid bacterium is deleted for genes or lacks functional genes or genes encoding polypeptides having an enzymatic activity of:

viii. lactate dehydrogenase (E.C 1.1.1.27 or E.C.1.1.1.28)

ix. phosphotransacetylase (E.C.2.3.1.8) and

x. bifunctional alcohol dehydrogenase (E.C. 1.1.1.1 and EC 1.2.1.10).

Figure 1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LacEF
Lactose-6P
LacG
Glucose
Galactose-6P
Glucose-6P
LacABC
LacD
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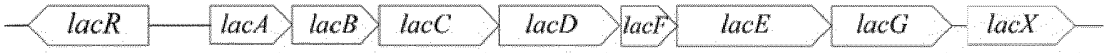
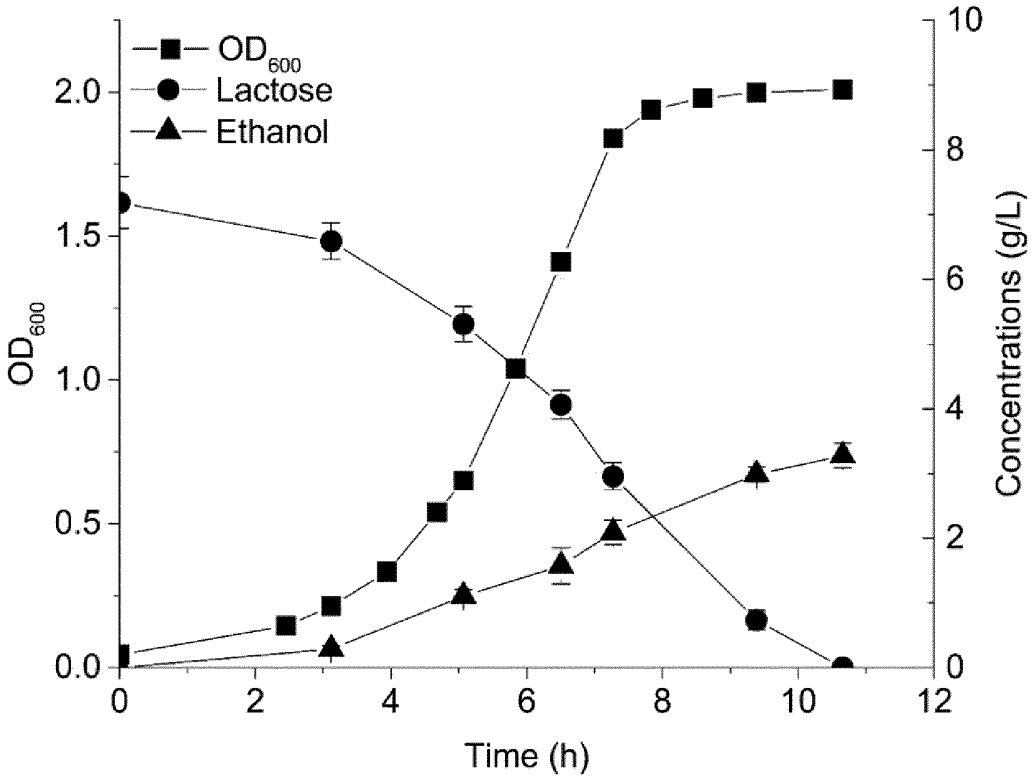
4 G3P
4 Ethanol
4 Ethanol
4 ADP
4 NAD+
ADHB
CSLH
AA metabolism
4 ATP
4 NADH
Lactate
4 Pyruvate
4 Acetaldehyde
LDH
PDC
PTA
ADHE
Acetate
Acetyl-CoA
Ethanol

Figure 2



Gene	EC number	Function	Length (AA)
lacA	EC 5.3.1.26	galactose-6-phosphate isomerase subunit	141
lacB	EC 5.3.1.26	galactose-6-phosphate isomerase subunit	171
lacC	EC 2.7.1.144	tagatose-6-phosphate kinase	310
lacD	EC 4.1.2.40	tagatose-1,6-bisphosphate aldolase	326
lacE	EC 2.7.1.69	PTS system, lactose-specific EIICB component	568
lacF	EC 2.7.1.69	PTS system, lactose-specific EIIA component	105
lacG	EC 3.2.1.85	phospho-β-D-galactosidase	468
lacX	EC 5.1.3.3	aldose 1-epimerase	299
lacR		lactose transport regulator	255

Figure 3


OD600
Lactose
Ethanol
OD600
Concentrations (g/L)
Time (h)
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0.5
1.0
1.5
2.0
0
2
4
6
8
10
12

Figure 4

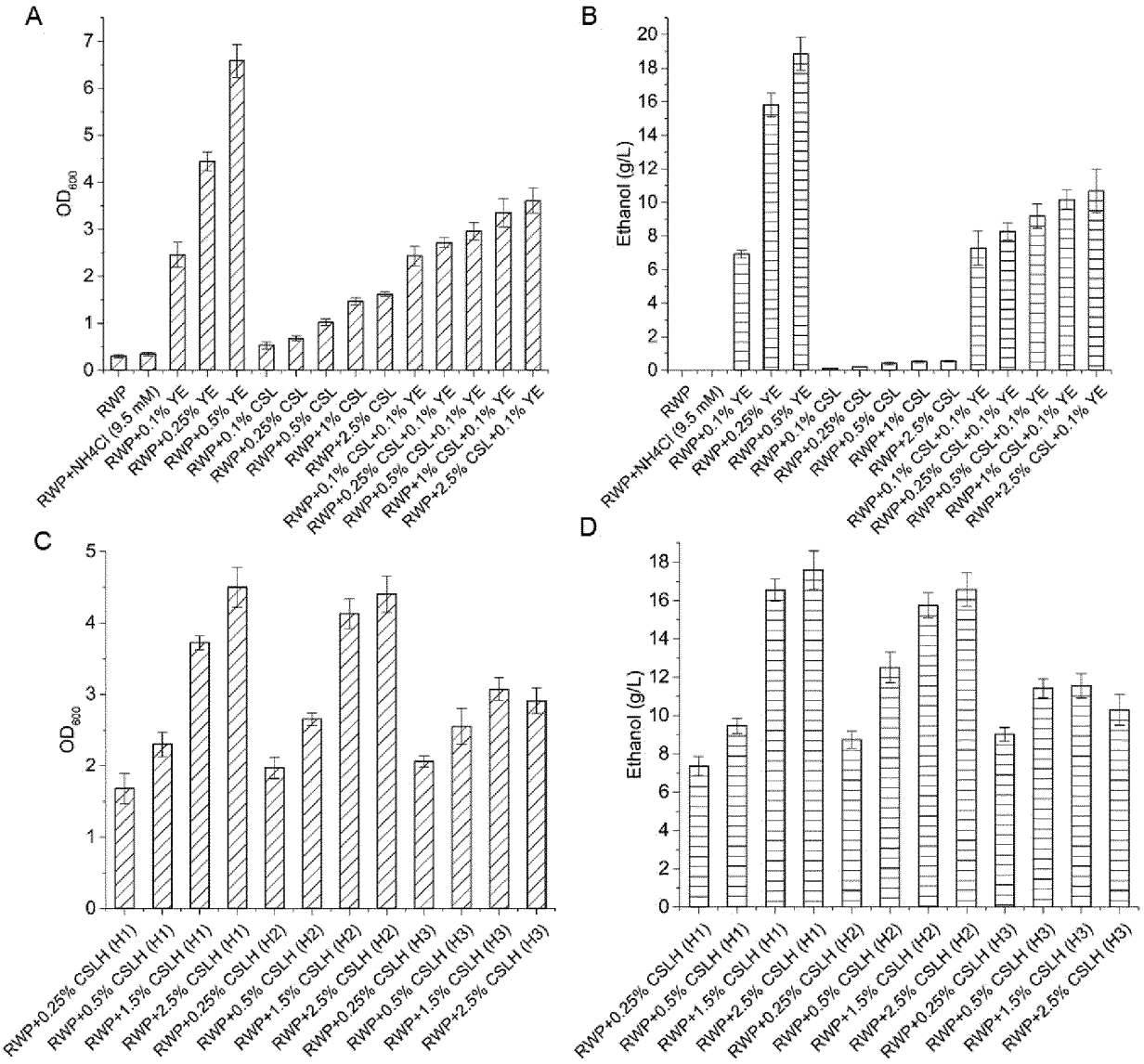

A
OD_{600}
RWP
RWP+NH4Cl (9.5 mM)
RWP+0.1% YE
RWP+0.25% YE
RWP+0.5% YE
RWP+0.1% CSL
RWP+0.25% CSL
RWP+0.5% CSL
RWP+1% CSL
RWP+2.5% CSL
RWP+0.1% CSL+0.1% YE
RWP+0.25% CSL+0.1% YE
RWP+0.5% CSL+0.1% YE
RWP+1% CSL+0.1% YE
RWP+2.5% CSL+0.1% YE
B
Ethanol (g/L)
C
OD_{600}
RWP+0.25% CSLH (H1)
RWP+0.5% CSLH (H1)
RWP+1.5% CSLH (H1)
RWP+2.5% CSLH (H1)
RWP+0.25% CSLH (H2)
RWP+0.5% CSLH (H2)
RWP+1.5% CSLH (H2)
RWP+2.5% CSLH (H2)
RWP+0.25% CSLH (H3)
RWP+0.5% CSLH (H3)
RWP+1.5% CSLH (H3)
RWP+2.5% CSLH (H3)
D
Ethanol (g/L)

Figure 5

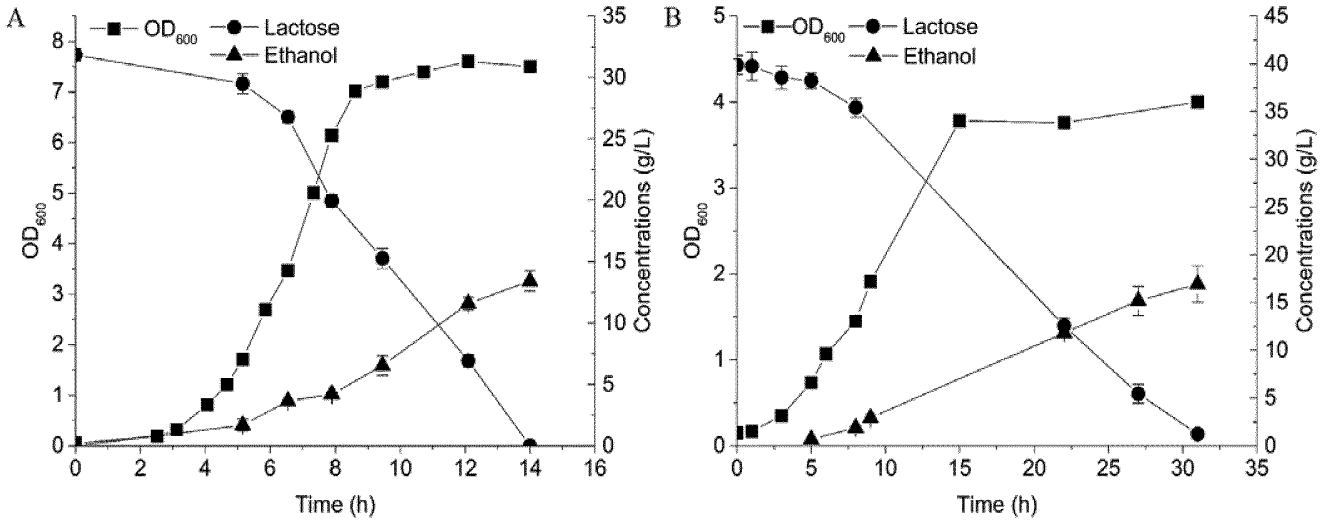

A
OD600
Lactose
Ethanol
OD600
Concentrations (g/L)
Time (h)
B
OD600
Lactose
Ethanol
OD600
Concentrations (g/L)
Time (h)

Figure 6

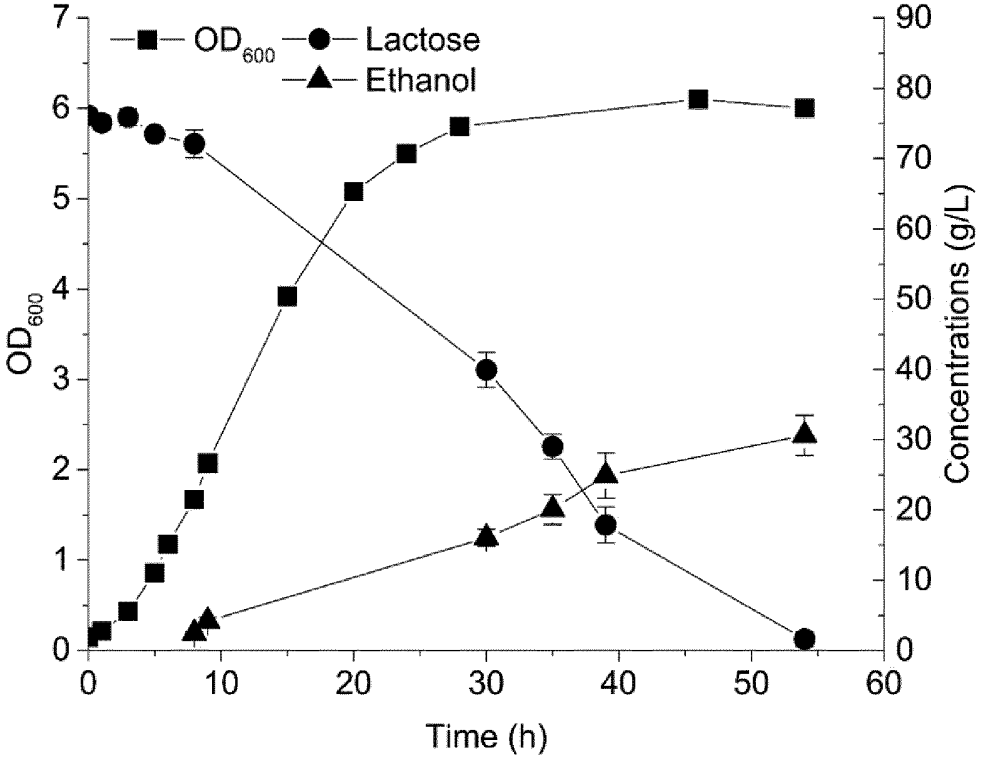


Figure 7

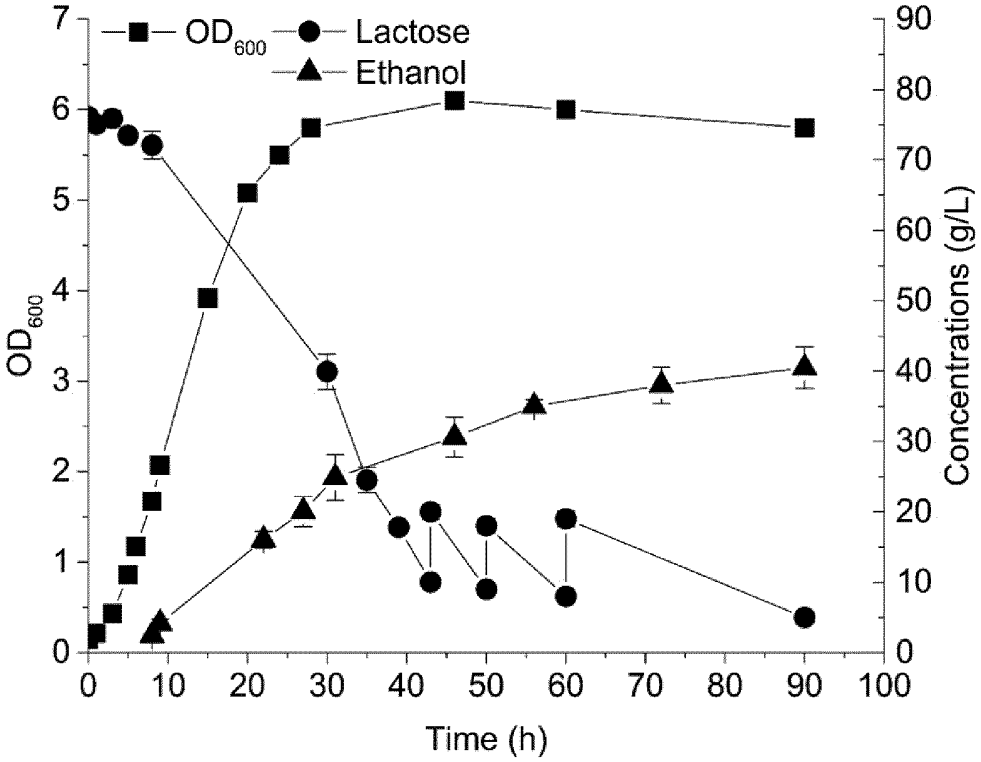
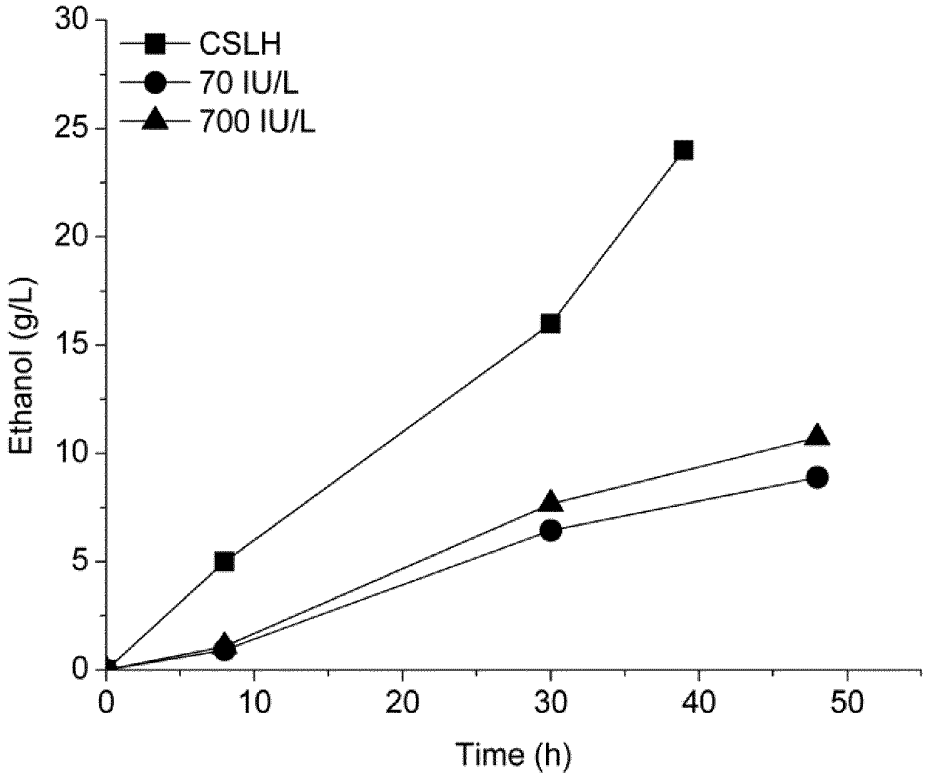


Figure 8


CSLH
70 IU/L
700 IU/L
Ethanol (g/L)
Time (h)
0
5
10
15
20
25
30
0
10
20
30
40
50

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/054347

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/06 C12N15/75
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, COMPENDEX, FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SOLEM ET AL: "Rewiring Lactococcus lactis for ethanol production", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 79, 2013, pages 2512-2518, XP002757325, cited in the application * See page 2515 (Figure 1) and page 2517 (Table 3/CS4435) *	1,3-13, 15

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[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 May 2017	16/05/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Korsner, Sven-Erik

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SILVA ET AL: "Fermentation of deproteinized cheese whey powder solutions to ethanol by engineered Saccharomyces cerevisiae: effect of supplementation with corn steep liquor and repeated-batch operation with biomass recycling by flocculation", JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, vol. 37, 2010, pages 973-982, XP019809576, * See page 973 (Abstract/CSL) and page 976 (CSL) *	1,3-13, 15
Y	LEITE ET AL: "Fermentation of sweet whey by recombinant Escherichia coli KO11", BRAZILIAN JOURNAL OF MICROBIOLOGY, vol. 31, 2000, pages 212-215, XP002757326, * See page 212 (left column/plasmids) and page 213 (right column/nitrogen source, protease) *	1,3-13, 15
Y	PRICE ET AL: "From meadows to milk to mucosa - adaptation of Streptococcus and Lactococcus species to their nutritional environments", FEMS MICROBIOLOGY REVIEWS, vol. 36, 2012, pages 949-971, XP002757327, * See page 952 (Figure 2) *	1,3-13, 15
Y	RUHDAL JENSEN ET AL: "Minimal requirements for exponential growth of Lactococcus lactis", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 59, 1993, pages 4363-4366, XP002902580, cited in the application * See page 4364 (Table 2) *	1,3-13, 15
Y	CRETENET ET AL: "Early adaptation to oxygen is key to the industrially important traits of Lactococcus lactis ssp. cremoris during milk fermentation", BMC GENOMICS, vol. 15, 2014, pages 1-15, XP021206658, * See page 11 (Methods/first paragraph -> pLP712) *	1,3-13, 15

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2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEGMANN ET AL: "Molecular characterization and structural instability of the industrially important composite metabolic plasmid pLP712", MICROBIOLOGY, vol. 158, 2012, pages 2936-2945, XP002757491, cited in the application * See page 2936 (Abstract) *	1,3-13, 15
Y	DE LIMA ET AL: "The use of response surface methodology in optimization of lactic acid production: focus on medium supplementation, temperature and pH control", FOOD TECHNOLOGY AND BIOTECHNOLOGY, vol. 48, 2010, pages 175-181, XP002757381, * See pages 178-179 (bridging paragraph) *	1,3-13, 15
A	DE FELICE ET AL: "Genetic structure of a novel biofuel-producing microorganism community", JOURNAL OF GENETICS, vol. 91, 2012, pages 183-191, XP035107872, * See pages 189-190 (Discussion) *	1-15
X,P	LIU ET AL: "A novel cell factory for efficient production of ethanol from dairy waste", BIOTECHNOLOGY FOR BIOFUELS, vol. 9, 26 February 2016 (2016-02-26), pages 1-11, XP002757328, * See in particular the Figures *	1-15
Y	DAHIYA ET AL: "Comparative analysis of bioethanol production from whey by different strains of immobilized thermotolerant yeast", INTERNATIONAL JOURNAL OF SCIENTIFIC AND RESEARCH PUBLICATIONS, vol. 2, 2012, pages 1-5, XP002769860, * See page 2 (C. Production of bioethanol/whey + corn steep liquor) *	1,3-13, 15
Y	GASPAR ET AL: "From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria", BIOTECHNOLOGY ADVANCES, vol. 31, 2013, pages 764-788, XP002769861, * See page 774 (Figure 4/Ethanol), page 775 (section 3.1.4), page 776 (Figure 5/Ethanol), and page 778 (Figure 6/Ethanol) *	1,3-13, 15

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